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I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002952835 for a patent by INTERNATIONAL FLOWER DEVELOPMENTS PTY LTD as filed on 16 September 2002.

WITNESS my hand this
Sixteenth day of August 2004

LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT
AND SALES

Regulation 3.2

International Flower Developments Pty Ltd

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Genetic sequences and uses therefor - II"

The invention is described in the following statement:

GENETIC SEQUENCES AND USES THEREFOR - II

FIELD OF THE INVENTION

The present invention relates generally to a genetic sequence encoding a polypeptide having an improved flavonoid 3',5'-hydroxylating activity and to the use of the genetic sequence and/or its corresponding polypeptide thereof such as in the manipulation of color in flower or other plant tissue. More particularly, the improved flavonoid 3',5'-hydroxylase (F3'5'H) has the ability to modulate DHK metabolism as well as the metabolism of other substrates such as DHQ, naringenin and eriodictyol. Even more particularly, the improved flavonoid F3'5'H (hereinafter referred to as "improved F3'5'H") of the present invention is isolated from pansy, salvia or sollya. Even yet more particularly, the present invention provides a genetic sequence encoding a polypeptide having improved F3'5'H activity when expressed in rose or gerbera or botanically related plants. The instant invention further relates to antisense and sense molecules or RNAi-inducing molecules corresponding to all or part of the subject genetic sequence or a transcript thereof as well as to genetically modified plants as well as cut flowers, parts and reproductive tissue from such plants. The present invention further relates to promoters which operate efficiently in plants such as rose, gerbera or botanically related plants.

BACKGROUND OF THE INVENTION

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Bibliographic details of references provided in the subject specification are listed at the end of the specification.

The flower or ornamental plant industry strives to develop new and different varieties of flowers and/or plants. An effective way to create such novel varieties is through the

manipulation of flower color. Classical breeding techniques have been used with some success to produce a wide range of colors for almost all of the commercial varieties of flowers and/or plants available today. This approach has been limited, however, by the constraints of a particular species' gene pool and for this reason it is rare for a single species to have the full spectrum of colored varieties. For example, the development of novel colored varieties of plants or plant parts such as flowers, foliage and stems would offer a significant opportunity in both the cut flower and ornamental markets. In the flower or ornamental plant industry, the development of novel colored varieties of major flowering species such as rose, chrysanthemum, tulip, lily, carnation, gerbera, orchid, lisianthus, begonia, torenia, geranium, petunia, nierembergia, pelargonium, impatiens and cyclamen would be of great interest. A more specific example would be the development of a blue rose or gerbera for the cut flower market.

In addition, the development of novel colored varieties of plant parts such as vegetables, fruits and seeds would offer significant opportunities in agriculture. For example, novel colored seeds would be useful as proprietary tags for plants. Furthermore modifications to flavonoids common to berries including grapes and their juices including wine have the potential to impart altered style characteristics of value to such fruit and byproduct industries.

Flower color is predominantly due to three types of pigment: flavonoids, carotenoids and betalains. Of the three, the flavonoids are the most common and contribute a range of colors from yellow to red to blue. The flavonoid molecules that make the major contribution to flower color are the anthocyanins, which are glycosylated derivatives of cyanidin and its methylated derivative peonidin, delphinidin and its methylated derivatives petunidin and malvidin and pelargonidin. Anthocyanins are localised in the vacuole of the epidermal cells of petals or the vacuole of the sub epidermal cells of leaves.

The flavonoid pigments are secondary metabolites of the phenylpropanoid pathway. The biosynthetic pathway for the flavonoid pigments (flavonoid pathway) is well established, (Holton and Cornish, *Plant Cell 7*: 1071-1083, 1995; Mol *et al.*, *Trends Plant Sci. 3*: 212–

217, 1998; Winkel-Shirley, *Plant Physiol. 126*: 485-493, 2001a; and Winkel-Shirley, *Plant Physiol. 127*: 1399-1404, 2001b) and is shown in Figures 1A and B. Three reactions and enzymes are involved in the conversion of phenylalanine to *p*-coumaroyl-CoA, one of the first key substrates in the flavonoid pathway. The enzymes are phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA (provided by the action of acetyl CoA carboxylase (ACC) on acetyl CoA and CO_2) with one molecule of *p*-coumaroyl-CoA. This reaction is catalysed by the enzyme chalcone synthase (CHS). The product of this reaction, 2',4,4',6', tetrahydroxy-chalcone, is normally rapidly isomerized by the enzyme chalcone flavanone isomerase (CHI) to produce naringenin. Naringenin is subsequently hydroxylated at the 3 position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

The pattern of hydroxylation of the B-ring of dihydrokaempferol (DHK) plays a key role in determining petal color. The B-ring can be hydroxylated at either the 3', or both the 3' and 5' positions, to produce dihydroquercetin (DHQ) or dihydromyricetin (DHM), respectively. Two key enzymes involved in this part of the pathway are flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase, both of the cytochrome P450 class of enzymes. Cytochrome P450 enzymes are widespread in nature and genes have been isolated and sequenced from vertebrates, insects, yeasts, fungi, bacteria and plants.

Flavonoid 3'-hydroxylase (F3'H) generally acts on DHK to produce DHQ and on naringenin to produce eriodictyol. Reduction and glycosylation of DHQ produces the cyanidin-glycoside based pigments which, in many plant species (for example *Rosa spp.*, *Dianthus spp.* and chrysanthemum), contribute to red and pink flower color.

Flavonoid 3', 5'-hydroxylase (F3'5'H) generally acts on DHK and DHQ to produce DHM and on naringenin and eriodictyol to produce pentahydroxyflavanone. Reduction and glycosylation of DHM produces the delphinidin-glycoside based pigments which, in many plant species (for example, *Petunia spp.*, *Viola spp.*, *Lisianthus spp.*, *Gentiana spp.*, *Sollya spp.*, *Salvia spp.*, *Clitoria spp.*, *Kennedia spp.*, *Campanula spp.*, *Lavandula spp.*, *Verbena*

spp., Torenia spp., Delphinium spp., Solanum spp., Cineraria spp., Vitis spp., Babiana stricta, Pinus spp., Picea spp., Larix spp., Phaseolus spp., Vaccinium spp., Cyclamen spp., Iris spp., Pelargonium sp., Liparieae, *Geranium spp., Pisum spp., Lathyrus spp., Catharanthus spp., Malvia spp., Mucuna spp., Vicia spp., Saintpaulia spp., Lagerstroemia spp., Tibouchina spp., Plumbago spp., Hypocalyptus spp., Rhododendron spp., Linum spp., Macroptilium spp., Hibiscus spp., Hydrangea spp., Cymbidium spp., Millettia spp., Hedysarum spp., Lespedeza spp., Asparagus spp. Antigonon spp., Pisum spp., Freesia spp., Brunella spp., Clarkia spp.*, etc.), contribute to purple and blue flower color. Many plant species such as roses, gerberas, chrysanthemums and carnations (excluding genetically modified carnations described in International Patent Application No. PCT/AU96/00296), do not produce delphinidin-based pigments because they lack a F3'5'H activity.

The next step in the pathway, leading to the production of the colored anthocyanins from the dihydroflavonols (DHK, DHQ, DHM), involves dihydroflavonol-4-reductase (DFR) leading to the production of the leucoanthocyanidins. The leucoanthocyanidins are subsequently converted to the anthocyanidins, pelargonidin, cyanidin and delphinidin. These flavonoid molecules are unstable under normal physiological conditions and glycosylation at the 3-position, through the action of glycosyltransferases, stabilizes the anthocyanidin molecule thus allowing accumulation of the anthocyanins. In general, the glycosyltransferases transfer the sugar moieties from UDP sugars to the flavonoid molecules and show high specificities for the position of glycosylation and relatively low specificities for the acceptor substrates (Seitz and Hinderer, Anthocyanins. In: *Cell Culture and Somatic Cell Genetics of Plants.* Constabel, F. and Vasil, I.K. (eds.), Academic Press, New York, USA, *5:* 49-76, 1988). Anthocyanins can occur as 3-monosides, 3-biosides and 3-triosides as well as 3, 5-diglycosides and 3, 7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose and xylose (Strack and Wray, In: *The Flavonoids - Advances in Research since 1986.* Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

Glycosyltransferases involved in the stabilisation of the anthocyanidin molecule include UDP glucose: flavonoid 3-glucosyltransferase (3GT), which transfers a glucose moiety from UDP glucose to the 3-*O*-position of the anthocyanidin molecule to produce anthocyanidin 3-*O*-glucoside.

In petunia and pansy (amongst others), anthocyanidin 3-*O*-glucoside are generally glycosylated by another glycosyltransferase, UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase (3RT), which adds a rhamnose group to the 3-*O*-bound glucose of the anthocyanin molecule to produce the anthocyanidin 3-rutinosides, and once acylated, can be further modified by UDP: glucose anthocyanin 5 glucosyltransferase (5GT). However, in roses (amongst others), the anthocyanidin 3-*O*-glucosides are generally glycosylated by another glycosyltransferase, UDP: glucose anthocyanin 5 glucosyltransferase (5GT) to produce anthocyanindin 3, 5 diglucosides.

Many anthocyanidin glycosides exist in the form of acylated derivatives. The acyl groups that modify the anthocyanidin glycosides can be divided into two major classes based upon their structure. The aliphatic acyl groups include malonic acid or succinic acid and the aromatic class include the hydroxy cinnamic acids such as *p*-coumaric acid, caffeic acid and ferulic acid and the benzoic acids such as *p*-hydroxybenzoic acid.

Methylation at the 3' and 5' positions of the B-ring of anthocyanidin glycosides can also occur. Methylation of cyanidin-based pigments leads to the production of peonidin. Methylation of the 3' position of delphinidin-based pigments results in the production of petunidin, whilst methylation of the 3' and 5' positions results in malvidin production.

In addition to the above modifications, pH of the vacuole or compartment where pigments are localised and copigmentation with other flavonoids such as flavonols and flavones can affect petal color. Flavonols and flavones can also be aromatically acylated (Brouillard and Dangles, In: *The Flavonoids -Advances in Research since 1986.* Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

The ability to control F3'5'H activity, or other enzymes involved in the flavonoid pathway, in flowering plants would provide a means to manipulate color of plant parts such as petals, fruit, leaves, sepals, seeds etc. Different colored versions of a single cultivar could thereby be generated and in some instances a single species would be able to produce a broader spectrum of colors.

Two nucleotide sequences (referred to herein as SEQ ID NO:1 and SEQ ID NO:3) encoding petunia F3'5'Hs have been cloned (see International Patent Application No. PCT/AU92/00334 and Holton *et al.*, 1993, *supra*). Although these sequences were efficient in modulating 3', 5' hydroxylation of flavonoids in petunia (see International Patent Application No. PCT/AU92/00334 and Holton *et al.*, 1993, *supra*), tobacco (see International Patent Application No. PCT/AU92/00334) and carnations (see International Patent Application No. PCT/AU96/00296), they were surprisingly unable to synthesize 3', 5'-hydroxylated flavonoids in roses. There is a need, therefore, to identify further genetic sequences encoding F3'5'Hs which efficiently modulate 3'5' hydroxylation of flavonoids such as anthocyanins in roses and other key commercial plant species.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc.

Genetic sequences encoding an improved F3'5'H have been identified and cloned from a number of species other than petunia. The genetic sequences of the present invention permit the modulation of expression of genes encoding this enzyme by, for example, *de novo* expression, over-expression, suppression, antisense inhibition, ribozyme activity, RNAi-induction or methylation-induction. The ability to control F3'5'H synthesis in plants and more specifically in roses permits modulation of the composition of individual anthocyanins as well as alteration of relative levels of flavonols and anthocyanins, thereby enabling the manipulation of color of plants such as petals, leaves, seeds, sepals, fruits etc.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding an F3'5'H or a derivative thereof wherein said F3'5'H or its derivative is capable of more efficient conversion of DHK to DHM in roses (and other species of commercial importance) than is the F3'5'H encoded by the nucleotide sequence set forth in SEQ ID NO:1 and SEQ ID NO:3.

Efficiency as used herein relates to the capability of the F3'5'H enzyme to convert its substrate DHK or DHQ into DHM in a rose cell (or plant cell of commercial importance). This provides the plant with a substrate (DHM) for other enzymes of the flavonoid pathway able to further modify this molecule *via*, for example, glycosylation, acylation and rhamnosylation, to produce various anthocyanins which contribute to the production of a

range of colors. The modulation of 3',5'-hydroxylated anthocyanins in rose is thereby enabled. Efficiency is conveniently assessed by one or more parameters selected from: extent of transcription, as determined by the amount of mRNA produced; extent of translation of mRNA, as determined by the amount of translation product produced; extent of enzyme activity as determined by the production of anthocyanin derivatives of DHQ or DHM; the extent of effect on flower color.

It has also been surprisingly discovered that certain combinations of promoter and F3'5'H gene sequences that were functional in carnation and petunia were not always functional in rose. Surprisingly, only a particular subset of promoter and F3'5'H gene sequence combinations resulted in 3'5'-hydroxylated flavonoids in rose flowers. These included F3'5'H sequences isolated from *Viola spp.*, *Salvia spp.* and *Sollya spp.* Furthermore, the *Viola* F3'5'H (or pansy F3'5'H) sequences were found to result in the highest accumulation of 3'5'-hydroxylated flavonoids in rose. The novel promoter and F3'5'H gene sequence combinations can be employed *inter alia* to modulate the color or flavour or other characteristics of plants or plant parts such as flowers, fruits, nuts, roots, stems, leaves or seeds. Thus, the present invention represents a new approach to developing plant varieties having altered color characteristics. Other uses include, for example, the production of novel extracts of F3'5'H transformed plants wherein the extract has use, for example, as a flavouring or food additive or health product or beverage or juice or coloring. Beverages may include but are not limited to wines, spirits, teas, coffee, milk and dairy products.

In a preferred embodiment, therefore, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding pansy F3'5'H, salvia F3'5'H or sollya F3'5'H or a functional derivative of the enzyme.

The nucleotide sequences encoding the F3'5'H pansy (SEQ ID NOs:9 and 11), F3'5'H salvia (SEQ ID NOs:13 and 15) and F3'5'H sollya (SEQ ID NO:17) are indicated in parentheses. A summary of the sequence identifiers is shown in Table 1.

Accordingly, another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:9 (pansy) or SEQ ID NO:11 (pansy) or SEQ ID NO:13 (salvia) or SEQ ID NO:15 (salvia) or SEQ ID NO:17 (sollya) or having at least about 50% similarity thereto or capable of hybridizing to the sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 under low stringency conditions.

The amino acid sequences of the preferred F3'5'H enzymes are set forth in SEQ ID NO:10 (pansy) or SEQ ID NO:12 (pansy) or SEQ ID NO:14 (salvia) or SEQ ID NO:16 (salvia) or SEQ ID NO:18 (sollya).

A further aspect of the present invention provides a method for producing a transgenic flowering plant capable of synthesizing an improved F3'5'H said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence which comprises a sequence of nucleotides encoding said improved F3'5'H under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence. The transgenic plant may thereby produce non-indigenous improved F3'5'H at elevated levels relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced indigenous or existing F3'5'H activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding an F3'5'H activity, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced indigenous or existing F3'5'H activity, said method

comprising altering the F3'5'H gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Still another aspect of the present invention contemplates a method for producing a transgenic flowering plant exhibiting altered inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence.

Still a further aspect of the present invention contemplates a method for producing a flowering plant exhibiting altered inflorescence properties, said method comprising alteration of the *F3'5'H* gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Even yet another aspect of the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding a F3'5'H or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule optionally transcribable where required to effect regulation of a F3'5'H, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, a F3'5'H, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell.

Even still another aspect of the present invention extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic

acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered inflorescence properties.

Even still another aspect of the present invention extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered aerial parts of the plant such as sepal, bract, petiole, peduncle, ovaries, anthers or stem properties.

Another aspect of the present invention contemplates the use of the extracts from transgenic plants or plant parts transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention and, in particular, the extracts from those transgenic plants when used as a flavouring or food additive or health product or beverage or juice or coloring.

A further aspect of the present invention is directed to recombinant forms of improved F3'5'H.

Another aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing an improved F3'5'H or down-regulating an indigenous F3'5'H enzyme in a plant.

Yet another aspect of the present invention is directed to a prokaryotic or eukaryotic organism carrying a genetic sequence encoding an improved F3'5'H extrachromasomally in plasmid form.

Still another aspect of the present invention extends to a recombinant polypeptide comprising a sequence of amino acids substantially as set forth in SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or an amino acid

sequence having at least about 50% similarity to SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or a derivative of said polypeptide.

The present invention further provides promoters which operate efficiently in plants such as rose and gerbera or botanically related plants. Such promoters include the rose CHS promoter, chrysanthemum CHS promoter and a CaMV promoter.

A summary of sequence identifiers used throughout the subject specification is provided in Table 1:

TABLE 1

Summary of sequence identifiers

SEQ ID NO:	NAME	DESCRIPTION
1	petunia *F3'5'H Hf1*.nt	petunia *F3'5'H* cDNA nucleotide seq (*Hf1*)
2	petunia *F3'5'H Hf1*.aa	translation of petunia *F3'5'H* (*Hf1*) seq
3	petunia *F3'5'H Hf2*.nt	petunia *F3'5'H* cDNA nucleotide seq (*Hf2*)
4	petunia *F3'5'H Hf2*.aa	translation of petunia *F3'5'H* (*Hf2*) seq
5	RoseCHS promoter	nucleotide sequence of rose chalcone synthase promoter fragment
6	D8 oligo#2	oligonucleotide to petunia PLTP (D8) gene
7	D8 oligo #4	oligonucleotide to petunia PLTP (D8) gene
8	chrysanCHSATG	oligonucleotide (#583) to chrysanthemum CHS promoter
9	BP#18.nt	pansy *F3'5'H* cDNA nucleotide seq (BP#18)
10	BP#18.aa	translation of pansy *F3'5'H* (BP#18) seq
11	BP#40.nt	pansy *F3'5'H* cDNA nucleotide seq (BP#40)
12	BP#40.aa	translation of pansy *F3'5'H* (BP#40) seq
13	Sal#2.nt	salvia *F3'5'H* cDNA nucleotide seq (Sal#2)
14	Sal#2.aa	translation of salvia *F3'5'H* (Sal#2) seq
15	Sal#47.nt	salvia *F3'5'H* cDNA nucleotide seq (Sal#47)
16	Sal#47.aa	translation of salvia *F3'5'H* (Sal#47) seq
17	Soll#5.nt	sollya *F3'5'H* cDNA nucleotide seq (Soll#5)
18	Soll#5.aa	translation of sollya *F3'5'H* (Soll#5) seq
19	FLS-Nco	oligonucleotide
20	BpeaHF2.nt	butterfly pea *F3'5'H* cDNA nucleotide seq
21	BpeaHF2.aa	translation of butterfly pea *F3'5'H* seq
22	Gen#48.nt	gentian *F3'5'H* cDNA nucleotide seq (Gen#48)
23	Gen#48.aa	translation of gentian *F3'5'H* (Gen#48) seq
24	PetD8 5'	nucletide sequence of OGB2.6 promoter fragment (petunia PLTP promoter)

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are schematic representations of the biosynthesis pathway for the flavonoid pigments. Figure 1A illustrates the general production of the anthocyanidin 3-glucosides that occur in most plants that produce anthocyanins. Figure 1B represents further modifications of anthocyanins that occur in petunia. Enzymes involved in the pathway have been indicated as follows: PAL = Phenylalanine ammonia-lyase; C4H = Cinnamate 4-hydroxylase; 4CL = 4-coumarate: CoA ligase; CHS = Chalcone synthase; CHI = Chalcone flavanone isomerase; F3H = Flavanone 3-hydroxylase; DFR = Dihydroflavonol-4-reductase; ANS = Anthocyanidin synthase, 3GT= UDP-glucose: flavonoid 3-*O*-glucosyltransferase; 3RT = UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase, AR-AT = Anthocyanidin-rutinoside acyltransferase, 5GT = Anthocyanin 5-glucosyltransferase; 3' OMT = Anthocyanin 3' *O*-methyltransferase, 3'5' OMT = Anthocyanin 3', 5' *O* -methyltransferase. Other abbreviations include: DHK = dihydrokaempferol, DHQ = dihydroquercetin, DHM = dihydromyricetin,

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, genetic sequences encoding improved F3'5'H have been identified, cloned and assessed. The recombinant genetic sequences of the present invention permit the modulation of expression of genes encoding this enzyme by, for example, *de novo* expression, over-expression, sense suppression, antisense inhibition, ribozyme, minizyme and DNAzyme activity, RNAi-induction or methylation-induction or other transcriptional or post-transcriptional silencing activities. The ability to control F3'5'H synthesis in plants permits modulation of the composition of individual anthocyanins as well as alteration of relative levels of flavonols and anthocyanins, thereby enabling the manipulation of petal color. Moreover, the present invention extends to plants and reproductive or vegetative parts thereof including flowers, seeds, vegetables, leaves, stems, etc., and more particularly, ornamental transgenic plants. The term transgenic also includes progeny plants from the primary transgenic plants.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding an improved F3'5'H or a functional derivative of the enzyme.

The present invention is described and exemplified herein by reference to the identification, cloning and manipulation of genetic sequences encoding an improved F3'5'H which, up to the present time, is a particularly convenient and useful F3'5'H enzyme for the practice of the invention herein disclosed. This is done, however, with the understanding that the present invention extends to all novel improved F3'5'H-like enzymes and their functional derivatives.

For convenience and by way of short hand notation only, reference herein to an improved F3'5'H enzyme includes F3'5'H acting on DHK as well as DHQ. Preferably, the improved F3'5'H enzyme is a pansy, salvia or sollya F3'5'H. The improved F3'5'H enzyme may also be considered to include a polypeptide or protein having an improved F3'5'H activity or F3'5'H-like activity. The latter encompasses derivatives having altered F3'5'H activities.

A preferred aspect of the present invention, therefore, is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding an improved or a functional mutant, derivative, part, fragment, homolog or analog of an improved F3'5'H.

By the term "nucleic acid molecule" is meant a genetic sequence in a non-naturally occurring condition. Generally, this means isolated away from its natural state or synthesized or derived in a non-naturally-occurring environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro*, including genomic DNA fragments recombinant or synthetic molecules and nucleic acids in combination with heterologous nucleic acids. It also extends to the genomic DNA or cDNA or part thereof encoding improved F3'5'H or a part thereof in reverse orientation relative to its own or another promoter. It further extends to naturally occurring sequences following at least a partial purification relative to other nucleic acid sequences.

The term "genetic sequences" is used herein in its most general sense and encompasses any contiguous series of nucleotide bases specifying directly, or *via* a complementary series of bases, a sequence of amino acids in an improved F3'5'H enzyme. Such a sequence of amino acids may constitute a full-length F3'5'H such as is set forth in SEQ ID NO: 10 (pansy) or SEQ ID NO:12 (pansy) or SEQ ID NO:14 (salvia) or SEQ ID NO:16 (salvia) or SEQ ID NO:18 (sollya) or an active truncated form thereof or may correspond to a particular region such as an N-terminal, C-terminal or internal portion of the enzyme. A genetic sequence may also be referred to as a sequence of nucleotides or a nucleotide sequence and includes a recombinant fusion of two or more sequences.

In accordance with the above aspects of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:9 (pansy) or SEQ ID NO:11 (pansy) or SEQ ID NO:13 (salvia) or SEQ ID NO:15 (salvia) or SEQ ID NO:17 (sollya) or having at least about 50% similarity thereto or capable of hybridizing to the sequence set forth in SEQ ID

NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 under low stringency conditions.

Table 1 provides a summary of the sequence identifiers.

Alternative percentage similarity encompassed by the present invention include at least about 60% or at least about 65% or at least about 70% or at least about 75% or at least about 80% or at least about 85% or at least about 90% or above, such as about 95% or about 96% or about 97% or about 98% or about 99%.

In a particularly preferred embodiment, there is provided an isolated nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:9 (pansy) or SEQ ID NO:11 (pansy) or SEQ ID NO:13 (salvia) or SEQ ID NO:15 (salvia) or SEQ ID NO:17 (sollya) or having at least about 50% similarity thereto or capable of hybridizing to the sequence set forth in SEQ ID NO:1 (petunia) or SEQ ID NO:3 (petunia) or complementary strands of either under low stringency conditions, wherein said nucleotide sequence encodes a polypeptide having an improved F3'5'H activity.

For the purposes of determining the level of stringency to define nucleic acid molecules capable of hybridizing to SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 reference herein to a low stringency includes and encompasses from at least about 0% to at least about 15% v/v formamide and from at least about 1M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions. Generally, low stringency is from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace the inclusion of formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which

includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% (Marmur and Doty, *J. Mol. Biol. 5:* 109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, *Eur. J. Biochem. 46:* 83, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 1.0% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 1.0% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

Another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or an amino acid sequence having at least about 50% similarity thereto.

The term similarity as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, similarity includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, similarity includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence

identity", "substantially similar" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids Res. 25:* 3389-3402, 1997). A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* ("Current Protocols in Molecular Biology" John Wiley & Sons Inc, 1994-1998, Chapter 15, 1998).

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched

positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software. Similar comments apply in relation to sequence similarity.

Alternative percentage similarity encompassed by the present invention include at least about 60% or at least about 65% or at least about 70% or at least about 75% or at least about 80% or at least about 85% or at least about 90% or above, such as about 95% or about 96% or about 97% or about 98% or about 99%.

The nucleic acid sequences contemplated herein also encompass oligonucleotides useful as genetic probes for amplification reactions or as antisense or sense molecules capable of regulating expression of the corresponding gene in a plant. An antisense molecule as used herein may also encompass a genetic construct comprising the structural genomic or cDNA gene or part thereof in reverse orientation relative to its or another promoter. It may also encompass a homologous genetic sequence. An antisense or sense molecule may also be directed to terminal or internal portions of the gene encoding a polypeptide having an improved F3'5'H activity or to combinations of the above such that the expression of the gene is reduced or eliminated.

With respect to this aspect of the invention, there is provided an oligonucleotide of 5-50 nucleotides having substantial similarity or complementarity to a part or region of a molecule with a nucleotide sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or a complementary form thereof. By substantial similarity or complementarity in this context is meant a hybridizable similarity under low, alternatively and preferably medium and alternatively and most preferably high stringency conditions specific for oligonucleotide hybridization (Sambrook *et al.*,

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA, 1989). Such an oligonucleotide is useful, for example, in screening for improved F3'5'H genetic sequences from various sources or for monitoring an introduced genetic sequence in a transgenic plant. The preferred oligonucleotide is directed to a conserved improved F3'5'H genetic sequence or a sequence conserved within a plant genus, plant species and/or plant variety.

In one aspect of the present invention, the oligonucleotide corresponds to the 5' or the 3' end of the improved F3'5'H genetic sequences. For convenience, the 5' end is considered herein to define a region substantially between the start codon of the structural gene to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural gene. It is clear, therefore, that oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such probes.

In one embodiment, the nucleic acid sequence encoding an improved F3'5'H or various functional derivatives thereof is used to reduce the level of an endogenous an improved F3'5'H (e.g. *via* co-suppression or antisense-mediated suppression) or other post-transcriptional gene silencing (PTGS) processes including RNAi or alternatively the nucleic acid sequence encoding this enzyme or various derivatives or parts thereof is used in the sense or antisense orientation to reduce the level of an improved F3'5'H. The use of sense strands, double or partially single stranded such as constructs with hairpin loops is particularly useful in inducing a PTGS response. In a further alternative, ribozymes, minizymes or DNAzymes could be used to inactivate target nucleic acid sequences.

Still a further embodiment encompasses post-transcriptional inhibition to reduce translation into polypeptide material. Still yet another embodiment involves specifically inducing or removing methylation.

Reference herein to the altering of an improved F3'5'H activity relates to an elevation or reduction in activity of up to 30% or more preferably of 30-50%, or even more preferably 50-75% or still more preferably 75% or greater above or below the normal endogenous or existing levels of activity. Such elevation or reduction may be referred to as modulation of an improved F3'5'H enzyme activity. Generally, modulation is at the level of transcription or translation of improved F3'5'H genetic sequences.

The nucleic acids of the present invention may be a ribonucleic acid or deoxyribonucleic acids, single or double stranded and linear or covalently closed circular molecules. Preferably, the nucleic acid molecule is cDNA. The present invention also extends to other nucleic acid molecules which hybridize under low, preferably under medium and most preferably under high stringency conditions with the nucleic acid molecules of the present invention and in particular to the sequence of nucleotides set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or a part or region thereof. In its most preferred embodiment, the present invention extends to a nucleic acid molecule having a nucleotide sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or to a molecule having at least 40%, more preferably at least 45%, even more preferably at least 55%, still more preferably at least 65%-70%, and yet even more preferably greater than 85% similarity at the level of nucleotide or amino acid sequence to at least one or more regions of the sequence set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 and wherein the nucleic acid encodes or is complementary to a sequence which encodes an enzyme having an improved F3'5'H activity. It should be noted, however, that nucleotide or amino acid sequences may have similarities below the above given percentages and yet still encode an improved F3'5'H activity and such molecules may still be considered in the scope of the present invention where they have regions of sequence conservation. The present invention further extends to nucleic acid molecules in the form of oligonucleotide primers or probes capable of hybridizing to a portion of the nucleic acid molecules contemplated above, and in particular those set forth in SEQ ID NO:9 or SEQ ID NO:11 or SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17, under low, preferably under medium and most preferably under high stringency conditions. Preferably the

portion corresponds to the 5' or the 3' end of the gene. For convenience the 5' end is considered herein to define a region substantially between the start codon of the structural genetic sequence to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural genetic sequence. It is clear, therefore, that oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such probes.

The term gene is used in its broadest sense and includes cDNA corresponding to the exons of a gene. Accordingly, reference herein to a gene is to be taken to include:-

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

The term gene is also used to describe synthetic or fusion molecules encoding all or part of an expression product. In particular embodiments, the term nucleic acid molecule and gene may be used interchangeably.

The nucleic acid or its complementary form may encode the full-length enzyme or a part or derivative thereof. By "derivative" is meant any single or multiple amino acid substitutions, deletions, and/or additions relative to the naturally occurring enzyme and which retains an improved F3'5'H activity. In this regard, the nucleic acid includes the naturally occurring nucleotide sequence encoding an improved F3'5'H or may contain single or multiple nucleotide substitutions, deletions and/or additions to said naturally occurring sequence. The nucleic acid of the present invention or its complementary form may also encode a "part" of the improved F3'5'H, whether active or inactive, and such a nucleic acid molecule may be useful as an oligonucleotide probe, primer for polymerase

chain reactions or in various mutagenic techniques, or for the generation of antisense molecules.

Reference herein to a "part" of a nucleic acid molecule, nucleotide sequence or amino acid sequence, preferably relates to a molecule which contains at least about 10 contiguous nucleotides or five contiguous amino acids, as appropriate.

Amino acid insertional derivatives of the improved F3'5'H of the present invention include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with Table 2.

TABLE 2 Suitable residues for amino acid substitutions

Original residue	Exemplary substitutions
Ala	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn; Glu
Glu	Asp
Gly	Pro
His	Asn; Gln
Ile	Leu; Val

Original residue	Exemplary substitutions
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile; Val
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu; Met

Where the improved F3'5'H is derivatized by amino acid substitution, the amino acids are generally replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like. Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.

The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis (Merrifield, *J. Am. Chem. Soc. 85:* 2149, 1964) and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13 mutagenesis. The manipulation of DNA sequence to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently described, for example, in Sambrook *et al.* (1989, *supra*).

Other examples of recombinant or synthetic mutants and derivatives of the improved F3'5'H enzyme of the present invention include single or multiple substitutions, deletions

and/or additions of any molecule associated with the enzyme such as carbohydrates, lipids and/or proteins or polypeptides.

The terms "analogs" and "derivatives" also extend to any functional chemical equivalent of an improved F3'5'H and also to any amino acid derivative described above. For convenience, reference to improved F3'5'H herein includes reference to any functional mutant, derivative, part, fragment, homolog or analog thereof.

The present invention is exemplified using nucleic acid sequences derived from pansy, salvia or sollya since this represents the most convenient and preferred source of material to date. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants or certain microorganisms. All such nucleic acid sequences encoding directly or indirectly an improved F3'5'H are encompassed by the present invention regardless of their source. Examples of other suitable sources of genes encoding improved F3'5'H include, but are not limited to *Vitis spp.*, *Babiana stricta*, *Pinus spp.*, *Picea spp.*, *Larix spp.*, *Phaseolus spp.*, *Vaccinium spp.*, *Cyclamen spp.*, *Iris spp.*, *Pelargonium spp.*, Liparieae, *Geranium spp.*, *Pisum spp.*, *Lathyrus spp.*, *Clitoria spp.*, *Catharanthus spp.*, *Malva spp.*, *Mucuna spp.*, *Vicia spp.*, *Saintpaulia spp.*, *Lagerstroemia spp.*, *Tibouchina spp.*, *Plumbago spp.*, *Hypocalyptus spp.*, *Rhododendron spp.*, *Linum spp.*, *Macroptilium spp.*, *Hibiscus spp.*, *Hydrangea spp.*, *Cymbidium spp.*, *Millettia spp.*, *Hedysarum spp.*, *Lespedeza spp.*, *Asparagus spp.* *Antigonon spp.*, *Freesia spp.*, *Brunella spp.*, *Clarkia spp.*, etc.

In accordance with the present invention, a nucleic acid sequence encoding an improved F3'5'H may be introduced into and expressed in a transgenic plant in either orientation thereby providing a means either to convert suitable substrates, if synthesized in the plant cell, ultimately into DHM, or alternatively to inhibit such conversion of metabolites by reducing or eliminating endogenous or existing improved F3'5'H activity. The production of these 3',5'-hydroxylated substrates will subsequently be converted to delphinidin-based pigments that will modify petal color and may contribute to the production of a bluer color. Expression of the nucleic acid sequence in the plant may be constitutive, inducible or

developmental and may also be tissue-specific. The word "expression" is used in its broadest sense to include production of RNA or of both RNA and protein. It also extends to partial expression of a nucleic acid molecule.

According to this aspect of the present invention, there is provided a method for producing a transgenic flowering plant capable of synthesizing an improved F3'5'H, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence which comprises a sequence of nucleotides encoding said improved F3'5'H under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence. The transgenic plant may thereby produce non-indigenous improved F3'5'H at elevated levels relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced indigenous or existing 3',5'-hydroxylase activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding an improved F3'5'H activity, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced indigenous or existing F3'5'H activity, said method comprising altering the *F3'5'H* gene through modification of the indigenous sequences via homologous recombination from an appropriately altered improved *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

As used herein an "indigenous" enzyme is one, which is native to or naturally expressed in a particular cell. A "non-indigenous" enzyme is an enzyme not native to the cell but

expressed through the introduction of genetic material into a plant cell, for example, through a transgene. An "endogenous" enzyme is an enzyme produced by a cell but which may or may not be indigenous to that cell.

In a preferred embodiment, the present invention contemplates a method for producing a transgenic flowering plant exhibiting altered inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence.

Alternatively, said method may comprise stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention or its complementary sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to alter the level of activity of the indigenous or existing F3'5'H. Preferably the altered level would be less than the indigenous or existing level of F3'5'H activity in a comparable non-transgenic plant. Without wishing to limit the present invention, one theory of mode of action is that reduction of the indigenous F3'5'H activity requires the expression of the introduced nucleic acid sequence or its complementary sequence. However, expression of the introduced genetic sequence or its complement may not be required to achieve the desired effect: namely, a flowering plant exhibiting altered inflorescence properties.

In a related embodiment, the present invention contemplates a method for producing a flowering plant exhibiting altered inflorescence properties, said method comprising alteration of the 3',5'-hydroxylase gene through modification of the indigenous sequences via homologous recombination from an appropriately altered improved *F3'5'H* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Preferably, the altered inflorescence includes the production of different shades of blue or red flowers or other colors, depending on the genotype and physiological conditions of the recipient plant.

Accordingly, the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding an improved F3'5'H or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule optionally transcribable where required to effect regulation of an improved F3'5'H, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, an improved F3'5'H, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell. By suitable plant is meant a plant capable of producing DHK and possessing the appropriate physiological properties required for the development of the color desired.

One skilled in the art will immediately recognise the variations applicable to the methods of the present invention, such as increasing or decreasing the expression of the enzyme naturally present in a target plant leading to differing shades of colors such as different shades of blue, purple or red.

The present invention, therefore, extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered inflorescence properties. The transgenic plants may contain an introduced nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an improved F3'5'H. Generally, the nucleic acid would be stably introduced into the plant genome, although the present invention also extends to the introduction of an improved *F3'5'H* nucleotide sequence within an autonomously-replicating nucleic acid sequence such as a DNA or RNA virus capable of replicating within the plant cell. The

invention also extends to seeds from such transgenic plants. Such seeds, especially if colored, are useful as proprietary tags for plants. Any and all methods for introducing genetic material into plant cells are encompassed by the present invention.

Another aspect of the present invention contemplates the use of the extracts from transgenic plants or plant parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention and, in particular, the extracts from those transgenic plants when used as a flavouring or food additive or health product or beverage or juice or coloring.

Plant parts contemplated by the present invention includes, but is not limited to flowers, fruits, nuts, roots, stems, leaves or seeds.

The extracts of the present invention may be derived from the plants or plant part in a number of different ways including chemical extraction or heat extraction or filtration or squeezing or pulverization.

The plant, plant part or extract can be utilized in any number of different ways such as for the production of a flavouring (e.g. a food essence), a food additive (e.g. a stabilizer, a colorant) a health product (e.g. an antioxidant, a tablet) a beverage (e.g. wine, spirit, tea) or a juice (e.g. fruit juice) or coloring (e.g. food coloring, fabric coloring, dye, paint).

A further aspect of the present invention is directed to recombinant forms of improved F3'5'H. The recombinant forms of the enzyme will provide a source of material for research to develop, for example, more active enzymes and may be useful in developing *in vitro* systems for production of colored compounds.

Still a further aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing an improved F3'5'H or down-regulating an indigenous F3'5'H enzyme in a plant.

Another aspect of the present invention is directed to a prokaryotic or eukaryotic organism carrying a genetic sequence encoding an improved F3'5'H extrachromasomally in plasmid form.

The present invention further extends to a recombinant polypeptide comprising a sequence of amino acids substantially as set forth in SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or an amino acid sequence having at least about 50% similarity to SEQ ID NO:10 or SEQ ID NO:12 or SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or a derivative of said polypeptide.

A "recombinant polypeptide" means a polypeptide encoded by a nucleotide sequence introduced into a cell directly or indirectly by human intervention or into a parent or other relative or precursor of the cell. A recombinant polypeptide may also be made using cell-free, *in vitro* transcription systems. The term "recombinant polypeptide" includes an isolated polypeptide or when present in a cell or cell preparation. It may also be in a plant or parts of a plant regenerated from a cell which produces said polypeptide.

A "polypeptide" includes a peptide or protein and is encompassed by the term "enzyme".

The recombinant polypeptide may also be a fusion molecule comprising two or more heterologous amino acid sequences.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

General methods

In general, the methods followed were as described in Sambrook *et al.* (1989, *supra*) or Plant Molecular Biology Manual (2nd edition), Gelvin and Schilperoot (eds), Kluwer Academic Publisher, The Netherlands, 1994 or Plant Molecular Biology Labfax, Croy (ed), Bios scientific Publishers, Oxford, UK, 1993.

The cloning vectors pBluescript and PCR script were obtained from Stratagene, USA. pCR7 2.1 was obtained from Invitrogen, USA.

E. coli transformation

The *Escherichia coli* strains used were:

DH5α

supE44, Δ (lacZYA-ArgF)U169, (ø80lacZΔM15), hsdR17(r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, deoR. (Hanahan, *J. Mol. Biol. 166:* 557, 1983)

XL1-Blue

supE44, hsdR17(r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, lac^-,[F'proAB, $lacI^q$, lacZΔM15, Tn10(tet^R)] (Bullock *et al., Biotechniques 5:* 376, 1987).

BL21-CodonPlus-RIL strain

ompT hsdS(Rb- mB-) *dcm*+ Tet^r *gal endA* Hte [*argU ileY leuW* Cam^r]

M15 *E. coli* is derived from *E.coli* K12 and has the phenotype Nal^s, Str^s, Rif^s, Thi^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+.

Transformation of the *E. coli* strains was performed according to the method of Inoue *et al.*, (*Gene 96:* 23-28, 1990).

Agrobacterium tumefaciens strains and transformations

The disarmed *Agrobacterium tumefaciens* strain used was AGL0 (Lazo *et al. Bio/technology 9:* 963-967, 1991).

Plasmid DNA was introduced into the *Agrobacterium tumefaciens* strain AGL0 by adding 5 μg of plasmid DNA to 100 μL of competent AGL0 cells prepared by inoculating a 50 mL LB culture (Sambrook *et al.*, 1989, *supra*) and incubation for 16 hours with shaking at 28°C. The cells were then pelleted and resuspended in 0.5 mL of 85% (v/v) 100 mM $CaCl_2$/15% (v/v) glycerol. The DNA-*Agrobacterium* mixture was frozen by incubation in liquid N_2 for 2 minutes and then allowed to thaw by incubation at 37°C for 5 minutes. The DNA/bacterial mix was then placed on ice for a further 10 minutes. The cells were then mixed with 1mL of LB (Sambrook *et al.*, 1989 *supra*) media and incubated with shaking for 16 hours at 28°C. Cells of *A. tumefaciens* carrying the plasmid were selected on LB agar plates containing appropriate antibiotics such as 50 μg/mL tetracycline or 100 μg/mL gentamycin. The confirmation of the plasmid in *A. tumefaciens* was done by restriction endonuclease mapping of DNA isolated from the antibiotic-resistant transformants.

DNA ligations

DNA ligations were carried out using the Amersham Ligation Kit or Promega Ligation Kit according to procedures recommended by the manufacturer.

Isolation and purification of DNA fragments

Fragments were generally isolated on a 1% (w/v) agarose gel and purified using the QIAEX II Gel Extraction kit (Qiagen) or Bresaclean Kit (Bresatec, Australia) following procedures recommended by the manufacturer.

Repair of overhanging ends after restriction endonuclease digestion

Overhanging 5' ends were repaired using DNA polymerase (Klenow fragment) according to standard protocols (Sambrook *et al.*, 1989 *supra*). Overhanging 3' ends were repaired using T4 DNA polymerase according to standard protocols (Sambrook *et al.*, 1989 *supra*).

Removal of phosphoryl groups from nucleic acids

Shrimp alkaline phosphatase (SAP) (USB) was typically used to remove phosphoryl groups from cloning vectors to prevent re-circularization according to the manufacturer's recommendations.

Polymerase Chain Reaction (PCR)

Unless otherwise specified, PCR conditions using plasmid DNA as template included using 2 ng of plasmid DNA, 100 ng of each primer, 2 µL 10 mM dNTP mix, 5 µL 10 x Taq DNA DNA polymerase buffer, 0.5 µL Taq DNA Polymerase in a total volume of 50 µL. Cycling conditions comprised an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 20 sec, 50°C for 30 sec and 72°C for 1 min with a final treatment at 72°C for 10 min before storage at 4°C.

PCRs were performed in a Perkin Elmer GeneAmp PCR System 9600.

^{32}P-Labelling of DNA Probes

DNA fragments (50 to 100 ng) were radioactively labelled with 50 µCi of [α-^{32}P]-dCTP using a Gigaprime kit (Geneworks). Unincorporated [α-^{32}P]-dCTP was removed by chromatography on Sephadex G-50 (Fine) columns or Microbiospin P-30 Tris chromatography columns (BioRad).

Plasmid Isolation

Single colonies were analyzed for inserts by inoculating LB broth (Sambrook *et al.*, 1989, *supra*) with appropriate antibiotic selection (e.g. 100 µg/mL ampicillin or 10 to 50 µg/mL tetracycline etc.) and incubating the liquid culture at 37°C (for *E. coli*) or 29°C (for *A. tumefaciens*) for ~16 hours with shaking. Plasmid DNA was purified using the alkali-lysis procedure (Sambrook *et al.*, 1989, *supra*) or using The WizardPlus SV minipreps DNA purification system (Promega) or Qiagen Plasmid Mini Kit (Qiagen). Once the presence of an insert had been determined, larger amounts of plasmid DNA were prepared from 50 mL overnight cultures using the alkali-lysis procedure (Sambrook *et al.*, 1989, *supra*) or

QIAfilter Plasmid Midi kit (Qiagen) and following conditions recommended by the manufacturer.

DNA Sequence Analysis

DNA sequencing was performed using the PRISM (trademark) Ready Reaction Dye Primer Cycle Sequencing Kits from Applied Biosystems. The protocols supplied by the manufacturer were followed. The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System 9600). Sequencing runs were generally performed by the Australian Genome Research Facility at The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) or in-house on an automated 373A DNA sequencer (Applied Biosystems).

Homology searches against Genbank, SWISS-PROT and EMBL databases were performed using the FASTA and TFASTA programs (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85(8):* 2444-2448, 1988) or BLAST programs (Altschul *et al., J. Mol. Biol. 215(3):* 403-410, 1990). Percentage sequence similarities were obtained using LALIGN program (Huang and Miller, *Adv. Appl. Math. 12:* 373-381, 1991) using default settings.

Multiple sequence alignments were produced using ClustalW (Thompson *et al., Nucleic Acids Research 22:* 4673-4680, 1994) using default settings.

EXAMPLE 2

Plant transformations

Petunia hybrida transformations (Sw63 x Skr4)

As described in Holton *et al.* (*Nature, 366*: 276-279, 1993) by any other method well known in the art.

Rosa hybrida transformations

As described in U.S. Patent Application No. 542,841 (PCT/US91/04412) or Robinson and Firoozabady (*Scientia Horticulturae, 55*: 83-99, 1993), Rout *et al.* (*Scientia Horticulturae,*

81: 201-238, 1999) or Marchant *et al.* (*Molecular Breeding 4*: 187-194, 1998) or by any other method well known in the art.

Cuttings of *Rosa hybrida* were generally obtained from Van Wyk and Son Flower Supply, Victoria.

Dianthus caryophyllus transformations

International Patent Application No. PCT/US92/02612 (carnation transformation). As described in International Patent Application No. PCT/AU96/00296 (Violet carnation), Lu *et al.* (*Bio/Technology 9*: 864-868, 1991), Robinson and Firoozabady (1993, *supra*) or by any other method well known in the art.

Cuttings of *Dianthus caryophyllus* cv. Kortina Chanel or Monte Lisa were obtained from Van Wyk and Son Flower Supply, Victoria.

EXAMPLE 3

Transgenic Analysis

Color coding

The Royal Horticultural Society's Color Chart (Kew, UK) was used to provide a description of observed color. They provide an alternative means by which to describe the color phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colors and should not be regarded as limiting the possible colors which may be obtained.

TLC and HPLC analysis

Generally as described in Brugliera *et al.* (*Plant J.* 5, 81-92, 1994).

Extraction of anthocyanidins

Prior to HPLC analysis, the anthocyanin and flavonol molecules present in petal and stamen extracts were acid hydrolysed to remove glycosyl moieties from the anthocyanidin

or flavonol core. Anthocyanidin and flavonol standards were used to help identify the compounds present in the floral extracts.

Anthocyanidins in the reaction mixture were analysed by HPLC *via* gradient elution using gradient conditions of 50%B to 60%B over 10 minutes, then 60% B for 10 minutes and finally 60% B to 100% B over 5 minutes where solvent A consisted of TFA: H_2O (5:995) and solvent B consisted of acetonitrile: TFA: H_2O (500:5:495). An Asahi Pac ODP-50 cartridge column (250 mm x 4.6 mm ID) was used for the reversed phase chromatographic separations. The flow rate was 1 mL/min and the temperature was 40°C. The detection of the anthocyanidin compounds was carried out using a Shimadzu SPD-M6A three dimensional detector at 400-650 nm.

The anthocyanidin peaks were identified by reference to known standards, *viz* delphinidin, petunidin, malvidin, cyanidin and peonidin

Stages of flower development

Petunia

Flowers of *Petunia hybrida* cv. Skr4 x Sw63 were generally harvested prior to anther dehiscence at the stage of maximum pigment accumulation.

Carnation

Dianthus caryophyllus flowers were harvested at developmental stages defined as follows:

Stage 1:	Closed bud, petals not visible.
Stage 2:	Flower buds opening: tips of petals visible.
Stage 3:	Tips of nearly all petals exposed. "Paint-brush stage".
Stage 4:	Outer petals at 45° angle to stem.
Stage 5:	Flower fully open.

Rose

Stages of *Rosa hybrida* flower development were defined as follows:

Stage 1: Unpigmented, tightly closed bud (10-12 mm high; 5 mm wide).

Stage 2: Pigmented, tightly closed bud (15 mm high ; 9 mm wide).

Stage 3: Pigmented, closed bud; sepals just beginning to open (20-25 mm high; 13-15 mm wide).

Stage 4: Flower bud beginning to open; petals heavily pigmented; sepals have separated (bud is 25-30 mm high and 18 mm wide).

Stage 5: Sepals completely unfolded; some curling. Petals are heavily pigmented and unfolding (bud is 30-33 mm high and 20 mm wide).

Anthocyanin/flavonol measurements by spectrophotometric measurements

Approximately 200mg of fresh petal tissue was added to 2 mL of methanol/1% (v/v) HCl and incubated for ~16 hours at 4°C. A 1 in 20 dilution (e.g. 50 µL made to 1000 µL) was then made and the absorbance at 350 nm and 530 nm was recorded.

The approximate flavonols and anthocyanin amounts (nmoles/gram) were then calculated according to the following formulae:

Anthocyanin content

$$\frac{(A_{530}\ /34{,}000)\ x\ \text{volume of extraction buffer (mL)}\ x\ \text{dilution factor}\ x\ 10^6}{\text{mass of petal tissue (grams)}}$$

Flavonol content

$$\frac{(A_{350}\ /14{,}300)\ x\ \text{volume of extraction buffer (mL)}\ x\ \text{dilution factor}\ x\ 10^6}{\text{mass of petal tissue (grams)}}$$

Northern analysis

Total RNA was isolated from petals or leaves using a Plant RNAeasy kit (QIAGEN) following procedures recommended by the manufacturer. For rose samples 1% (w/v) PVP was added to the extraction buffer.

RNA samples (5 μg) were electrophoresed through 2.2 M formaldehyde/1.2% w/v agarose gels using running buffer containing 40 mM morpholinopropanesulphonic acid (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA (pH 8.0). The RNA was transferred to Hybond-N membrane filters (Amersham) as described by the manufacturer.

RNA blots were probed with ^{32}P-labelled fragments. Prehybridization (1 hour at 42°C) and hybridization (16 hours at 42°C) of the membrane filters were carried out in 50% v/v formamide, 1 M NaCl, 1% w/v SDS, 10% w/v dextran sulphate. The membrane filters were generally washed in 2 x SSC, 1% w/v SDS at 65°C for between 1 to 2 hours and then 0.2 x SSC, 1% w/v SDS at 65°C for between 0.5 to 1 hour. Membrane filters were generally exposed to Kodak XAR film with an intensifying screen at -70°C for 22 hours.

EXAMPLE 4

Introduction of chimeric petunia F3'5'H genes into rose

As described in the introduction, the pattern of hydroxylation of the B-ring of the anthocyanidin molecule plays a key role in determining petal color. The production of the dihydroflavonol DHM, leads to the production of the purple/blue delphinidin-based pigments in plants such as petunia. The absence of the *F3'5'H* activity has been correlated with the absence of blue flowers in many plant species such as *Rosa*, *Gerbera*, *Antirrhinum*, *Dianthus* and *Dendranthema*.

Based on success in producing delphinidin-based pigments in a mutant petunia line (Holton *et al.*, 1993a, *supra* and International Patent Application No. PCT/AU92/00334), in tobacco flowers (International Patent Application No. PCT/AU92/00334) and in carnation flowers (International Patent Application No. PCT/AU96/00296), chimeric

petunia *F3'5'H* genes were also introduced into roses in order to produce novel delphinidin-based pigments and modify flower color.

Preparation of chimeric petunia F3'5'H gene constructs

TABLE 3 bbreviations used in construct preparations

ABBREVIATION	DESCRIPTION
AmCHS 5'	1.2 kb promoter fragment from the *Antirrhinum majus* CHS gene (Sommer and Saedler, *Mol Gen. Gent.*, 202: 429-434, 1986)
CaMV 35S	~0.2 kb incorporating *Bgl*II fragment containing the promoter region from the Cauliflower Mosaic Virus 35S gene (*CaMV 35S*) (Franck *et al.*, *Cell* 21: 285-294, 1980, Guilley *et al.*, *Cell*, 30: 763-773. 1982)
chrysCHS 5'	promoter region from a chalcone synthase gene from chrysanthemum
GUS	β-glucuronidase coding sequence (Jefferson, *et al.*, *EMBO J.* 6: 3901-3907, 1987)
Mac	Hybrid promoter consisting of the promoter from the *mas* gene and a *CaMV 35S* enhancer region (Comai *et al.*, *Plant Mol. Biol.* 15: 373-381, 1990)
mas/35S	Hybrid promoter consisting of promoter region from *CaMV 35S* gene with enhancing elements from the promoter of mannopine synthase gene of *Agrobacterium tumefaciens* (Klee *et al.*, *Bio/Technology 3:* 637-642, 1985)
mas 5'	Promoter region from mannopine synthase gene of *A. tumefaciens*
mas 3'	Terminator region from mannopine synthase gene of *A. tumefaciens*
nos 5'	Promoter region from nopaline synthase gene of *A. tumefaciens* (Depicker *et al.*, *J Mol. and Appl. Genetics* 1: 561-573, 1982)
nos 3'	Terminator region from nopaline synthase gene of *A. tumefaciens* (Depicker *et al.*, 1982, *supra*)
nptII	Kanamycin-resistance gene (encodes neomycin phosophotransferase which deactivates aminoglycoside antibiotics such as kanamycin, neomycin and G418)
ocs 3'	Terminator region from octopine synthase gene of *A. tumefaciens* (described in Klee *et al.*, 1985, *supra*)
petD8 5'	~3.2kb promoter region from phospholipid transfer protein gene (D8) of *Petunia hybrida* (Holton, Isolation and characterization of petal specific genes from *Petunia hybrida*. PhD thesis, University of Melbourne, Australia, 1992) (SEQ ID NO: 24)

ABBREVIATION	DESCRIPTION
petD8 3'	terminator region from phospholipid transfer protein gene (D8) of *Petunia hybrida* cv. OGB (Holton, 1992, *supra*)
long petFLS 5'	~4.0kb fragment containing the promoter region from flavonol synthase gene of *P. hybrida*
short petFLS 5'	~2.2kb fragment containing the promoter region from flavonol synthase gene of *P. hybrida*
petFLS 3'	~0.95kb fragment containing the terminator region from flavonol synthase gene of *P. hybrida*
petHf1	Petunia *Hf1* cDNA clone (Holton *et al.*, 1993, *supra*) (SEQ ID NO: 1)
petHf2	Petunia *Hf2* cDNA clone (Holton *et al.*, 1993, *supra*) (SEQ ID NO: 3)
petRT 5'	Promoter region of an anthocyanidin-3- glucoside rhamnosyltransferase from *P. hybrida* (Brugliera, Characterization of floral specific genes isolated from *Petunia hybrida*. RMIT, Australia. PhD thesis, 1994)
petRt 3'	Terminator region of a anthocyanidin-3- glucoside rhamnosyltransferase (3RT) gene from *P. hybrida* (Brugliera, 1994, *supra*)
RoseCHS 5'	~2.8kb fragment containing the promoter region from chalcone synthase gene (CHS) of *Rosa hybrida* (SEQ ID: 5)
SuRB	Chlorsulfuron-resistance gene (encodes Acetolactate Synthase) with its own terminator from *Nicotiana tabacum* (Lee *et al.*, *EMBO J.* 7: 1241-1248, 1988)

Binary vector constructs containing petunia F3'5'H cDNA fragments under the control of various promoters were prepared (Table 4).

TABLE 4 Summary of *chimeric*chimeric petunia *F3'5'H* gene cassettes contained in binary vector constructs used in the transformation of roses, carnations and petunias (see Table 3 for an explanation of abbreviations).

PLASMID	*F3'5'H* CASSETTE	SELECTABLE MARKER CASSETTE
pCGP1452	*AmCHS 5': petHf1: petD8 3'*	*CaMV 35S: SuRB*
pCGP1453	*Mac: petHf1: mas 3'*	*CaMV 35S: SuRB*
pCGP1457	*petD8 5': petHf1: petD8 3'*	*CaMV 35S: SuRB*
pCGP1461	*short petFLS 5': petHf1: petFLS 3'*	*CaMV 35S: SuRB*
pCGP1616	*petRT 5': petHf1: nos 3'*	*CaMV 35S: SuRB*
pCGP1638	*CaMV 35S: petHf1: ocs 3'*	*CaMV 35S: SuRB*
pCGP1623	*mas 35S: petHf1: ocs 3'*	*CaMV 35S: SuRB*

PLASMID	*F3'5'H* CASSETTE	SELECTABLE MARKER CASSETTE
pCGP1860	*RoseCHS 5': petHf1: nos 3'*	*CaMV 35S: SuRB*
pCGP2123	*CaMV 35S: petHf2: ocs 3'*	*CaMV 35S: SuRB*

Isolation of petunia F3'5'H cDNA clones (Hf1 and Hf2)

The isolation and characterisation of cDNA clones of petunia *F3'5'H* (*Hf1* and *Hf2* contained in pCGP601 and pCGP175 respectively) (SEQ ID NO:1 and SEQ ID NO:3, respectivley) have been described in International Patent Application No. PCT/AU92/00334 and Holton *et al.* (1993, *supra*).

Construction of pCGP1452 (AmCHS 5': Hf1: petD8 3'binary)

The plasmid pCGP1452 contains a *chimeric* petunia *F3'5'H* (*Hf1*) gene under the control of a promoter fragment from the *Antirrhinum majus* chalcone synthase gene (*CHS)* (Sommer and Saedler, 1986, *supra*) with a terminator fragment from the petunia phospholipid transfer protein (PLTP) gene (*petD8 3'*) (Holton, 1992, *supra*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S: SuRB* gene of the Ti binary vector, pWTT2132 (DNA Plant Technologies, USA = DNAP).

The Ti binary vector pWTT2132

The Ti binary vector plasmid pWTT2132 (DNAP) contains a chimeric gene comprised of a *CaMV 35S* promoter sequence (Franck *et al.*, 1980, *supra*), ligated with the coding region and terminator sequence for acetolactate synthase (ALS) gene from the *SuRB* locus of tobacco (Lee *et al.*, 1988, *supra*). An ~60bp 5' untranslated leader sequence from the petunia chlorophyll a/b binding protein gene (Cab 22 gene) (Harpster *et al.*, *MGG*, 212: 182-190, 1988) is included between the *CaMV 35S* promoter fragment and the *SuRB* sequence.

Construction of pCGP725 (AmCHS 5': Hf1: petD8 3' in pBluescript)

A chimeric petunia *F3'5'H* gene under the control *Antirrhinum majus CHS* (*AmCHS 5'*) promoter with a petunia PLTP terminator (*petD8 3'*) was constructed by cloning the 1.6kb *BclI/Fsp*I petunia *F3'5'H* (*Hf1*) fragment from pCGP602 (Holton *et al.*, 1993, *supra*)

between a 1.2 kb *Antirrhinum majus CHS* gene fragment 5' to the site of translation initiation (Sommer and Saedler, 1986, *supra*) and a 0.7 kb *SmaI/XhoI* PLTP fragment (*petD8 3'*) from pCGP13ΔBam (Holton, 1992, *supra*), 3' to the deduced stop codon. The resulting plasmids in a pBluescript II KS (Stratagene, USA) backbone vector were designated pCGP725 and pCGP726 (the only difference between each being the orientation of the expression cassette with respect to the *lacZ* region).

Construction of pCGP485 and pCGP1452 (AmCHS 5': Hf1: petD8 3'binary vectors)

The chimeric *F3'5'H* gene from pCGP725 was cloned into the Ti binary vector pCGN1547 containing an *nptII* selectable marker gene cassette (McBride and Summerfelt *Plant Molecular Biology 14:* 269-276, 1990) to create pCGP485. A 3.5 kb fragment containing the *AmCHS 5'*: *Hf1*: *petD8 3'* cassette was released upon digestion of pCGP485 with the restriction endonuclease *Pst*I. The overhanging ends were repaired and the purified 3.5 kb fragment was ligated with *Sma*I ends of the Ti binary vector, pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The plasmid was designated as pCGP1452.

Plant transformation with pCGP1452

The T-DNA contained in the binary vector plasmid pCGP1452 was introduced into rose, carnation and petunia *via Agrobacterium*-mediated transformation.

Construction of pCGP1453 (Mac: Hf1: mas 3' binary)

The plasmid pCGP1453 contains a chimeric petunia *F3'5'H* (*Hf1*) gene under the control of a *Mac* promoter (Comai *et al.*, 1990, *supra*) with a terminator fragment from the mannopine synthase gene of *Agrobacterium* (*mas 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette of the Ti binary vector, pWTT2132 (DNAP).

A 3.9 kb fragment containing the *Mac: Hf1: mas 3'* cassette was released from the plasmid pCGP628 (described in International Patent Application No. PCT/AU94/00265) upon digestion with the restriction endonuclease *Pst*I. The overhanging ends were repaired and the purified fragment was ligated with *Sma*I ends of pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The plasmid was designated as pCGP1453.

Plant transformation with pCGP1453

The T-DNA contained in the binary vector plasmid pCGP1453 was introduced into rose, carnation and petunia *via Agrobacterium*-mediated transformation.

Construction of pCGP1457 (petD8 5': Hf1: pet D8 3' binary vector)

The plasmid pCGP1457 contains a chimeric petunia *F3'5'H* (*Hf1*) gene under the control of a promoter fragment from the petunia *PLTP* gene (*petD8 5'*) with a terminator fragment from the petunia *PLTP* gene (*petD8 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* gene of the Ti binary vector, pWTT2132 (DNAP).

Isolation of petunia D8 genomic clone

Preparation of P. hybrida cv. OGB (Old Glory Blue) genomic library in λ2001

A genomic DNA library was constructed from *Petunia hybrida* cv. OGB DNA in the vector λ2001 (Karn *et al., Gene 32:* 217-224, 1984) using a *Sau*3A partial digestion of the genomic DNA as described in Holton, 1992 *(supra)*. Screening of the OGB genomic library for the petunia D8 gene was as described in Holton, 1992. *supra.*

Isolation of D8 genomic clone OGB2.6

PCR was performed in order to find a non-mutant genomic clone representing D8. Oligo #2 (5' to 3' GTTCTCGAGGAAAGATAATACAAT) (SEQ ID NO:6) and Oligo #4 (5' to 3' CAAGATCGTAGGACTGCATG) (SEQ ID NO:7) were used to amplify D8 gene fragments,

across the intron region, using 4 µL of phage suspension from the clones isolated from the primary screening of the OGB genomic library. The reactions were carried out in a total volume of 50 µL containing 1 x Amplification buffer (Cetus), 0.2 mM dNTP mix, <1 µg of template DNA, 50 pmoles of each primer and 0.25 µL of Taq polymerase (5 units/µL - Cetus). The reaction mixtures were overlaid with 30 µL of mineral oil and temperature cycled using a Gene Machine (Innovonics). The reactions were cylced 30 times using the following conditions: 94°C for 1 minute, 55°C for 50 seconds, 72°C for 2 minutes. One quarter of each PCR reaction was run on an agarose gel using TAE running buffer.

Three clones, λOGB-2.4, λOGB-2.5, and λOGB-2.6, gave fragments of approximately 1 kb whereas the mutant clone, λOGB-3.2 (described in Holton, 1992, *supra*), had produced a product of 1.25 kb. The λOGB-2.6 clone was chosen for further analysis.

pCGP382

The genomic clone, λOGB-2.6, contained a single 3.9 kb *Xba*I fragment that hybridized with the D8 cDNA. This *Xba*I fragment was isolated and purified and ligated with the *Xba*I ends of pBluescriptII KS- (Stratagene, USA). Restriction mapping of this clone revealed an internal *Pst*I site 350 bp from the 3′ end. However, the mutant clone, pCGP13, had an internal *Pst*I near the ATG of the coding region (approximately 1.5 kb from its 3′ end). The difference in the position of the *Pst*I site in both clones suggested that the λOGB-2.6 *Xba*I fragment did not contain the whole genomic sequence of D8. A Southern blot was performed on *Pst*I digested λOGB-2.6 DNA, and a fragment of 2.7 kb was found to hybridize with the D8 cDNA. Restriction endonuclease mapping confirmed that this fragment contained the 3′ coding region and flanking sequences.

In order to obtain a fragment containing the whole D8 genomic sequence, a number of cloning steps were undertaken. The λOGB-2.6 *Pst*I fragment of 2.7 kb was purified and ligated with *Pst*I ends of pBluescriptII KS- (Stratagene, USA). The resultant clone was digested with *Xba*I to remove the 350 bp *Pst*I/*Xba*I fragment. This fragment was replaced by the 3.9 kb *Xba*I fragment from λOGB-2.6 to produce the plasmid pCGP382.

A 3.2 kb fragment containing the promoter region from the *D8 2.6* gene in pCGP382 was released upon digestion with the restriction endonucleases *Hin*DIII and *Nco*I. The fragment was purified and ligated with the 4.8 kb *NcoI/Hin*DIII fragment of pJB1 (Bodeau, Molecular and genetic regulation of Bronze-2 and other maize anthocyanin genes. Dissertation, Stanford University, USA, 1994) to produce pCGP1101 containing a *petD85': GUS: nos 3'* cassette.

A 1.6 kb petunia *Hf1* fragment was released from the plasmid pCGP602 (Holton *et al.*, 1993a, supra) (SEQ ID NO:1) upon digestion with the restriction endonucleases *Bsp*HI and *Bam*HI. The fragment was purified and ligated with the 6.2 kb *NcoI/Bam*HI fragment of pCGP1101 to produce pCGP1102 containing a *petD8 5': Hf1: nos 3'* expression cassette.

A 0.75 kb *Bam*HI *petD8 3'* fragment (Holton, 1992, *supra*) was purified from the plasmid pCGP13ΔBamHI and ligated with *Bam*HI/*Bgl*II ends of pCGP1102 to produce the plasmid pCGP1107 containing a *petD8 5': Hf1: petD8 3'* expression cassette.

The plasmid pCGP1107 was firstly linearised upon digestion with the restriction endonuclease *Xba*I. The overhanging ends were repaired and then the 5.3 kb fragment containing the *petD8 5': Hf1: petD8 3'* expression cassette was released upon digestion with the restriction endonuclease *Pst*I. The fragment was purified and ligated with *Sma*I/*Pst*I ends of the binary vector pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The plasmid was designated as pCGP1457.

Plant transformation with pCGP1457

The T-DNA contained in the binary vector plasmid pCGP1457 was introduced into rose, carnation and petunia via *Agrobacterium*-mediated transformation.

Construction of pCGP1461 (short petFLS 5': Hf1: pet FLS 3' binary vector)

The plasmid pCGP1461 contains a chimeric petunia *F3'5'H* (*Hf1*) gene under the control of a promoter fragment from the petunia flavonol synthase (*FLS*) gene (*short petFLS 5'*) with a terminator fragment from the petunia *FLS* gene (*petFLS 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* gene of the Ti binary vector, pWTT2132.

Isolation of petunia FLS gene

Preparation of P. hybrida cv. Th7 genomic library

A *P. hybrida* cv. Th7 genomic library was prepared according to Sambrook *et al.* (1989, *supra*) using a*Sau*3A partial digestion of the genomic DNA. The partially digested DNA was cloned into EMBL-3 lambda vector (Stratagene, USA).

The Th7 genomic DNA library was screened with ^{32}P-labelled fragments of a petunia *FLS* cDNA clone (Holton *et al.*, *Plant J. 4:* 1003-1010, 1993b) using high stringency conditions.

Two genomic clones (*FLS2* and *FLS3*) were chosen for further analysis and found to contain sequences upstream of the putative initiating methionine of the petunia *FLS* coding region with *FLS2* containing a longer promoter region than *FLS3*.

A 6 kb fragment was released upon digestion of the genomic clone *FLS2* with the restriction endonuclease *Xho*I. The fragment containing the short petunia *FLS* gene was purified and ligated with *Xho*I ends of pBluescript SK (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP486.

A 9 kb fragment was released upon digestion of the genomic clone *FLS3* with the restriction endonuclease *Xho*I. The fragment containing the petunia *FLS* gene was purified and ligated with *Xho*I ends of pBluescript SK (Stratagene, USA). Correct insertion of the

fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP487.

A 2.2 kb petunia *FLS* promoter fragment upstream from the putative translational initiation site was released from the plasmid pCGP487 upon digestion with the restriction endonucleases *Xho*I and *Pst*I. The fragment generated was purified and ligated with *XhoI/PstI* ends of pBluescript II KS+ (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP717.

A 0.95 kb petunia *FLS* terminator fragment downstream from the putative translational stop site was released from the plasmid pCGP487 upon digestion with the restriction endonucleases *Hin*dIII and *Sac*I. The fragment generated was purified and ligated with *Hin*dIII/*Sac*I ends of pBluescript II KS+ (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP716.

Construction of pCGP493 (short petFLS 5':petFLS3' expression cassette)

A 1.8 kb fragment containing the short petunia *FLS* promoter fragment was amplified by PCR using the plasmid pCGP717 as template and the T3 primer (Stratagene, USA) and an FLS-*Nco* primer (5' AAA ATC GAT ACC ATG GTC TTT TTT TCT TTG TCT ATA C 3') (SEQ ID NO:19). The PCR product was digested with the restriction endonucleases *Xho*I and *Cla*I and the purified fragment was ligated with *XhoI/Cla*I ends of pCGP716. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP493.

Construction of pCGP497 (short petFLS 5': Hf1: petFLS3' expression cassette)

The petunia *F3'5'H* (*Hf1*) cDNA clone was released from the plasmid pCGP601 (described above) upon digestion with the restriction endonucleases *Bsp*HI and *Fsp*I. The *Bsp*HI recognition sequence encompasses the putative translation initiating codon and the *Fsp*I

recognition sequence commences 2 bp downstream from the stop codon. The *Hf1* fragment generated was purified and ligated with *Cla*I (repaired ends)/*Nco*I ends of the plasmid pCGP493. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP497.

Construction of pCGP1461 (short petFLS 5': Hf1: petFLS3' binary vector)

The plasmid pCGP497 was linearised upon digestion with the restriction endonuclease *Sac*I. The overhanging ends were repaired and a 4.35 kb fragment containing the *short petFLS 5': Hf1: petFLS3'* gene expression cassette was released upon digestion with the restriction endonuclease *Kpn*I. The fragment generated was purified and ligated with *Pst*I (ends repaired)/*Kpn*I ends of the Ti binary vector pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette of pWTT2132 was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The resulting plasmid was designated as pCGP1461.

Plant transformation with pCGP1461

The T-DNA contained in the binary vector plasmid pCGP1461 was introduced into rose, carnation and petunia via *Agrobacterium*-mediated transformation.

Construction of pCGP1616 (petRT 5': Hf1: nos 3' binary vector)

The plasmid pCGP1616 contains a chimeric petunia *F3'5'H* (*Hf1*) gene under the control of a promoter fragment from the *P. hybrida 3RT* gene (*petRT 5'*) (Brugliera, 1994, *supra*) with a terminator fragment from the nopaline synthase gene (*nos 3'*) of *Agrobacterium* (Depicker, *et al.*, 1982, *supra*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* gene of the Ti binary vector, pWTT2132 (DNAP).

P. hybrida cv. Th7 genomic DNA library construction in EMBL3

A *Petunia hybrida* cv. Th7 genomic library was prepared according to Sambrook *et al.* 1989, *supra* using a *Sau*3A partial digestion of the genomic DNA. The partially digested DNA was cloned into EMBL-3 lambda vector (Stratagene, USA). Screening of the Th7 genomic library for the petunia *3RT* gene was as described in Brugliera, 1994, *supra*).

A 3 kb fragment containing the *petRT 5': Hf1: nos 3'* cassette was released from the plasmid pCGP846 (described in Brugliera, 1994, *supra*) upon digestion with the restriction endonucleases *Pst*I and *Bam*HI. The purified fragment was ligated with *Pst*I/*Bam*HI ends of pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The plasmid was designated as pCGP1616.

Plant transformation with pCGP1616

The T-DNA contained in the binary vector plasmid pCGP1616 was introduced into rose, carnation and petunia via *Agrobacterium*-mediated transformation.

Construction of pCGP1623 (mas/35S: Hf1: ocs 3')

The plasmid pCGP1623 contains a chimeric petunia *F3'5'H* (*Hf1*) gene under the control of the expression cassette contained in pKIWI101 (Klee *et al.*, 1985, *supra*) consisting of a promoter fragment from the cauliflower mosaic virus *35S* gene (*CaMV 35S*) with an enhancing sequence from the promoter of the mannopine synthase gene (*mas*) of *Agrobacterium* and a terminator fragment from the octopine synthase gene of *Agrobacterium* (*ocs 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* gene of the Ti binary vector, pWTT2132 (DNAP).

The ~1.6 kb fragment of the petunia *F3'5'H Hf1* cDNA clone contained in the plasmid pCGP1303 was released upon digestion with the restriction endonucleases *Bsp*HI and *Sma*I. The *Hf1* fragment was purified and ligated with a ~5.9 kb *Nco*I/*Eco*RI (repaired ends) fragment of pKIWI101 (Klee *et al.*, 1985, *supra*) to produce the plasmid pCGP1619.

A partial digest of the plasmid pCGP1619 with the restriction endonuclease *Xho*I released a 4.9 kb fragment containing the *mas/35S: Hf1: ocs 3'* expression cassette. The fragment was purified and ligated with *Sal*I ends of pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The plasmid was designated as pCGP1623.

Plant transformation with pCGP1623

The T-DNA contained in the binary vector plasmid pCGP1623 was introduced into rose, carnation and petunia *via Agrobacterium*-mediated transformation.

Construction of pCGP1638 (CaMV 35S: Hf1: ocs 3' binary vector)

The plasmid pCGP1638 contains a chimeric petunia *F3'5'H* (*Hf1*) gene under the control of a *CaMV 35S* promoter (*CaMV 35S*) with an octopine synthase terminator (*ocs 3'*). A ~60 bp 5' untranslated leader sequence from the petunia chlorophyll a/b binding protein gene (*Cab 22* gene) (Harpster *et al.*, 1988, *supra*) is included between the *CaMV 35S* promoter fragment and the *Hf1* cDNA clone. The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette of the Ti binary vector, pWTT2132.

Plant transformation with pCGP1638

The T-DNA contained in the binary vector plasmid pCGP1638 was introduced into rose, carnation and petunia *via Agrobacterium*-mediated transformation.

Construction of pCGP1860 (RoseCHS 5': Hf1: nos 3' binary vector)

The plasmid pCGP1860 contains a chimeric petunia *F3'5'H* (*Hf1*) gene under the control of a promoter fragment from the chalcone synthase gene of *Rosa hybrida* (*RoseCHS 5'*) with a terminator fragment from the nopaline synthase gene of *Agrobacterium* (*nos 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette of the Ti binary vector, pWTT2132 (DNAP).

Isolation of Rose CHS promoter

A rose genomic DNA library was prepared from genomic DNA isolated from young leaves of *Rosa hybrida* cv. Kardinal.

The Kardinal genomic DNA library was screened with ^{32}P-labelled fragment of rose *CHS* cDNA clone contained in the plasmid pCGP634. The rose *CHS* cDNA clone was isolated by screening of a petal cDNA library prepared from RNA isolated from petals of *Rosa hybrida* cv Kardinal (Tanaka *et al., Plant Cell Physiol. 36:* 1023-1031, 1995) using a petunia *CHS* cDNA fragment as probe (clone *1F11* contained in pCGP701, described in Brugliera *et al.*, 1994, *supra*). Conditions are as described in Tanaka *et al.*, 1995 (*supra*).

A rose genomic clone (*roseCHS20*□) was chosen for further analysis and found to contain ~6.4 kb of sequence upstream of the putative initiating methionine of the rose *CHS* coding region.

An ~6.4 kb fragment upstream from the translational initiation site was cloned into pBluescript KS (-) (Statagene) and the plasmid was designated as pCGP1114.

The plasmid pCGP1114 was digested with the restriction endonucleases *Hind*III and *Eco*RV to release a 2.7-3.0kb fragment which was purified and ligated with the *Hind*III/*Sma*I ends of pUC19 (New England Biolabs). Correct insertion of the rose *CHS* promoter fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1116. The DNA sequence of the rose *CHS* promoter fragment was determined using pCGP1116 as template (SEQ ID NO:5).

Construction of pCGP197 (RoseCHS 5': GUS : nos 3' in pUC18 backbone)

An ~3.0 kb fragment containing the rose chalcone synthase promoter (*RoseCHS 5'*) was released from the plasmid pCGP1116 upon digestion with the restriction endonucleases *Hind*III and *Asp*718. The fragment was purified and ligated with a *Hind*II/*Asp*718 fragment

from pJB1 (Bodeau, 1994, *supra*) containing the vector backbone, β-glucoronidase (*GUS*) and *nos 3'* fragments. Correct insertion of the rose *CHS* promoter fragment upstream of the *GUS* coding sequence was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP197.

Construction of pCGP1303 (Hf1 in pUC19 backbone)

The petunia *F3'5'H* cDNA clone contained in the plasmid pCGP601 (a homolog of the *F3'5'H* contained in pCGP602 described in Holton *et al.*, 1993a, *supra*) included 64 bp of 5' untranslated sequence and 141 bp of 3' untranslated sequence including 16 bp of the poly A tail. The plasmid pCGP601 was firstly linearized by digestion with the restriction endonuclease *Bsp*HI. The ends were repaired and the *Hf1* cDNA clone was released upon digestion with the restriction endonuclease *Fsp*I. The *Bsp*HI recognition sequence encompasses the putative translation initiating codon and the *Fsp*I recognition sequence commences 2 bp downstream from the stop codon. The 1.8 kb fragment containing the *Hf1* cDNA clone was purified and ligated with repaired *Eco*RI ends of pUC19 (New England Biolabs). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1303.

Construction of pCGP200 (RoseCHS 5': Hf1: nos 3' in pUC18 backbone)

A 1.8 kb fragment containing the petunia *F3'5'H* (*Hf1*) fragment was released from the plasmid pCGP1303 upon digestion with the restriction endonucleases *Bsp*HI and *Sac*I. The *Hf1* fragment was purified and ligated with *Nco*I/*Sac*I ends of pCGP197. Correct insertion of the *Hf1* fragment between the rose *CHS* promoter and *nos 3'* fragments was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP200.

Construction of pCGP1860 (RoseCHS 5': Hf1: nos 3' in a binary vector)

An ~4.9 kb fragment containing the *RoseCHS 5': Hf1: nos 3'* cassette was released from the plasmid pCGP200 upon digestion with the restriction endonuclease *Bgl*II. The

fragment was purified and ligated with *Bam*HI ends of the Ti binary vector, pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette of pWTT2132 was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The resulting plasmid was designated as pCGP1860.

Plant transformation with pCGP1860

The T-DNA contained in the binary vector plasmid pCGP1860 was introduced into rose, carnation and petunia *via Agrobacterium*-mediated transformation.

Construction of pCGP2123 (CaMV 35S: Hf2: ocs 3' binary vector)

The plasmid pCGP2123 contains a chimeric petunia *F3'5'H* (*Hf2*) gene under the control of a CaMV35S promoter with a terminator fragment from the octopine synthase gene of *Agrobacterium* (*ocs 3'*). The chimeric petunia *F3'5'H* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassetteof the Ti binary vector, pCGP1988.

Construction ofpCGP1988 (a derivative of the Ti binary vector, pWTT2132)

The binary vector pCGP1988 is based on Ti binary vector pWTT2132 (DNAP) but contains the multi-cloning site from pNEB193 (New England Biolabs). The plasmid pNEB193 was firstly linearized by digestion with the restriction endonuclease *Eco*RI. The overhanging ends were repaired and the multi-cloning fragment was released upon digestion with the restriction endonuclease *Pst*I. The fragment was purified and ligated with *Sal*I (ends repaired)/*Pst*I ends of the Ti binary vector pWTT2132 (DNAP). Correct insertion of the multi-cloning fragment into pWTT2132 was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The resulting plasmid was designated as pCGP1988.

Construction of pCGP2000 (CaMV 35S promoter fragment in pBluescript)

The plasmid pCGP2000 was an intermediate plasmid containing the cauliflower mosaic virus (CaMV) 35S promoter fragment in a pBluescript SK (Stratagene, USA) backbone.

The *CaMV 35S* promoter fragment from pKIW101 (Klee *et al.*, 1985, *supra*) was released upon digestion with the restriction endonucleases *Xba*I and *Pst*I. The ~0.35kb fragment generated was purified and ligated with *XbaI/Pst*I ends of the vector pBluescript SK. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated as pCGP2000.

Construction of pCGP2105 (CaMV 35S 5' and ocs 3' fragments in pBluescript)

The plasmid pCGP2105 contained a *CaMV 35S* promoter fragment along with a terminator fragment from the octopine synthase gene of *Agrobacterium* (*ocs 3'*) both from pKIWI101 (Klee *et al.*, 1985, *supra*).

The *ocs* 3' fragment from pKIWI101 (Klee *et al.*, 1985, *supra*) was isolated by firstly digesting the plasmid pKIW101 with the restriction endonuclease *Eco*RI, followed by repair of the overhanging ends, and finally by digestion with the restriction endonuclease *Xho*I to release a 1.6 kb fragment. This fragment was then ligated with *Hinc*II/*Xho*I ends of pCGP2000. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated pCGP2105.

Construction of pCGP2109 (CaMV 35S: Hf2: ocs 3' cassette in pBluescript)

The plasmid pCGP2109 contained the *CaMV 35S: Hf2: ocs 3'* cassette in a pBluescript backbone.

The 1.8 kb petunia *F3'5'H Hf2* cDNA clone was released from pCGP175 (Holton *et al.*, 1993a, *supra*) upon digestion with the restriction endonucleases *Xba*I and *Ssp*I. The overhanging ends were repaired and the purified fragment was ligated with *Pst*I (ends repaired)/*Eco*RV ends of pCGP2105 (described above). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated pCGP2109.

Construction of pCGP2123 (CaMV 35S: Hf2: ocs 3' cassette binary vector)

The *CaMV 35S: Hf2: ocs 3'* cassette was released from pCGP2109 upon digestion with the restriction endonucleases *Asp*718 and *Xba*I. The overhanging ends were repaired and the resultant ~3.8 kb fragment was purified and ligated with repaired ends of *Asp*718 of the Ti binary vector, pCGP1988. Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The plasmid was designated as pCGP2123.

Plant transformation with pCGP2123

The T-DNA contained in the binary vector plasmid pCGP2123 was introduced into rose, carnation and petunia *via Agrobacterium*-mediated transformation.

EXAMPLE 5

Analysis of transgenic roses

Athough over 250 transgenic Kardinal roses were produced (Table 5) none produced flowers with a change in color. TLC and/or HPLC analysis failed to detect accumulation of any delphinidin pigments. Subsequent Northern analysis on RNA isolated from petal tissue of these transgenic roses revealed either no detectable intact petunia *F3'5'H* (*Hf1* or *Hf2*) transcripts, or in some cases (see footnotes), degraded transcripts. Hybridization of the same membranes with the selectable marker gene cassette (*SuRB*) or with an endogenous rose *CHS* cDNA probe revealed discrete hybridizing transcripts suggesting that the total RNA isolated was intact and confirming the transgenic nature of the lines.

TABLE 5 Results of transgenic analysis of rose petals transformed with the T-DNA from various petunia *F3'5'H* (*Hf1* or *Hf2*) gene expression cassettes.

Plasmid	*F3'5'H* cassette	Transgenics	Delphinidin	Northern
pCGP1452	*AmCHS 5': petHf1: petD8 3'*	34	0/28	0/34[1]
pCGP1453	*Mac: petHf1: mas 3'*	16	0/14	0/13[2]
pCGP1457	*petD8 5': petHf1: petD8 3'*	11	0/11	0/11

Plasmid	*F3'5'H* cassette	Transgenics	Delphinidin	Northern
pCGP1461	*short petFLS 5': petHf1: petFLS 3'*	11	0/11	0/11
pCGP1616	*petRT 5': petHf1: nos 3'*	4	0/4	0/4
pCGP1623	*mas/35S: petHf1: ocs 3'*	27	0/20	0/12[3]
pCGP1638	*CaMV 35S: petHf1: ocs 3'*	22	0/14	0/14
pCGP1860	*RoseCHS 5': petHf1: nos 3'*	15	0/13	0/13
pCGP2123	*CaMV 35S: petHf2: ocs 3'*	40	0/26	0/10

Transgenics = number of transgenics produced

Delphinidin = number of transgenic lines with accumulating delphinidin (by TLC or HPLC)/total number of events analyzed

Northern = number of transgenic lines with detectable intact Hf1 or Hf2 transcripts/total numer of events analyzed

[1] = Degraded transcripts were detected in 5 of the 34 analyzed

[2] = Degraded transcripts were detected in 8 of the 13 analyzed

[3] = Degraded transcripts were detected in 8 of the 12 analyzed

EXAMPLE 6

Evaluation of promoters in roses

Development of GUS gene expression cassettes.

From the results obtained with *Hf1* and *Hf2* constructs (detailed above) (Table 5) it was unclear which expression cassettes were functional in rose petals. Therefore, a number of promoters were linked to the β-glucuronidase reporter gene (*GUS*) (Jefferson *et al.*, 1987, *supra*) and introduced into roses in an attempt to identify expression cassettes that function well in rose flowers.

A summary of the promoters evaluated and transcript levels obtained is given in Table 6.

TABLE 6 List of *GUS* chimeric gene expression cassettes evaluated in roses

Construct number	Expression cassette	Selectable marker gene cassette	Backbone
pCGP1307	*petD8 5': GUS: petD8 3'*	*mas 5': nptII :mas 3'*	pCGN1548
pCGP1506	*long petFLS 5': GUS: petFLS 3'*	*nos 5': nptII: nos 3'*	pBIN19
pCGP1626	*chrysCHS 5': GUS: petRT 3'*	*CaMV 35S: SuRB*	pWTT2132
pCGP1641	*petRT 5': GUS: petRT 3'*	*CaMV 35S: SuRB*	pWTT2132
pCGP1861	*RoseCHS 5': GUS: nos 3'*	*CaMV 35S: SuRB*	pWTT2132
pCGP1953	*AmCHS 5': GUS: petD8 3'*	*CaMV 35S: SuRB*	pWTT2132
pWTT2084	*CaMV 35S: GUS: ocs 3'*	*CaMV 35S: SuRB*	pWTT2132

Construction of pCGP1307 (petD8 5': GUS: petD8 3' binary vector)

The plasmid pCGP1307 contains a chimeric *GUS* gene under the control of a promoter and terminator fragment from the petunia *PLTP* gene (*petD8 5'* and *petD8 3'*, respectively). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *mas 5': nptII: mas 3'* selectable marker gene cassette of the Ti binary vector pCGN1548 (McBride and Summerfelt, 1990, *supra*).

The *nos 3'* fragment from pCGP1101 (see Example 4) was replaced with the 0.75 kb *petD8 3'* fragment (Holton, 1992, *supra*) to produce the plasmid pCGP1106 containing a *petD8 5': GUS: petD8 3'* expression cassette.

The 5.3 kb fragment containing the *petD8 5': GUS: petD8 3'* expression cassette was released from the plasmid pCGP1106 upon digestion with the restriction endonucleases *Hin*DIII and *Pst*I. The fragment was purified and ligated with *Hin*DIII/*Pst*I ends of the Ti binary vector, pCGN1548. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from gentamycin-resistant transformants. The resulting plasmid was designated as pCGP1307.

Plant transformation with pCGP1307

The T-DNA contained in the binary vector plasmid pCGP1307 was introduced into rose *via Agrobacterium*-mediated transformation.

Construction of pCGP1506 (long petFLS 5': GUS: petFLS 3' binary vector)

The plasmid pCGP1506 contains a chimeric *GUS* gene under the control of promoter and terminator fragments from the petunia flavonol synthase gene (*petFLS 5'* and *petFLS 3', respectively)*. The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *nos 5': nptII: nos 3'* selectable marker gene cassette of the Ti binary vector pBIN19 (Bevan, *Nucleic Acids Res 12:* 8711-8721, 1984).

A 4 kb long petunia *FLS* promoter fragment upstream from the putative translational initiation site was released from the plasmid pCGP486 (described in Example 4) upon digestion with the restriction endonucleases *Xho*I and *Pst*I. The fragment generated was purified and ligated with *Xho*I/*Pst*I ends of pBluescript II KS+ (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP715.

Construction of pCGP494 (long petFLS 5':petFLS3' expression cassette)

A 4.0 kb fragment containing the long petunia *FLS* promoter fragment was amplified by PCR using the plasmid pCGP715 as template and the T3 primer (Stratagene, USA) and an FLS-*Nco* primer (5' AAA ATC GAT ACC ATG GTC TTT TTT TCT TTG TCT ATA C 3') (SEQ ID NO:19). The PCR product was digested with the restriction endonucleases *Xho*I and *Cla*I and the purified fragment was ligated with *Xho*I/*Cla*I ends of pCGP716. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP494.

Construction of pCGP496 (long petFLS 5': GUS: petFLS3' expression cassette)

The *GUS* coding sequence from the plasmid pJB1 (Bodeau, 1994, *supra*) was released upon digestion with the restriction endonucleases *Nco*I and *Sma*I. The *GUS* fragment generated was purified and ligated with *Cla*I (repaired ends)/*Nco*I ends of the plasmid pCGP494. Correct insertion of the fragment was established by restriction endonuclease

analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP496.

Construction of pCGP1506 (long petFLS 5': GUS: petFLS3' binary vector)

The plasmid pCGP496 was firstly linaerised upon digestion with the restriction endonuclease *Xho*I. The overhanging ends were repaired and a 6.7 kb fragment containing the *long petFLS 5': GUS: petFLS3'* gene expression cassette was released upon digestion with the restriction endonuclease *Sac*I. The fragment generated was purified and ligated with *Bam*HI(repaired ends)/*Sac*I ends of the Ti binary vector pBIN19. Correct insertion of the fragment in a tandem orientation with respect to the *nos 5': nptII: nos 3'* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from kanamycin-resistant transformants. The resulting plasmid was designated as pCGP1506.

Plant transformation with pCGP1506

The T-DNA contained in the binary vector plasmid pCGP1506 was introduced into rose *via Agrobacterium*-mediated transformation.

Construction of pCGP1626 (chrysCHS 5': GUS: petRT 3' binary vector)

The plasmid pCGP1626 contains a chimeric *GUS* gene under the control of promoter fragment from the chalcone synthase gene of chrysanthemum (*chrysCHS 5'*) and a terminator fragment from the 3RT gene of petunia (*petRT 3'*) (Brugliera, 1994, *supra*). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pWTT2132 (DNAP).

Isolation of chrysanthemum CHS promoter

A chrysanthemum genomic DNA library was prepared from genomic DNA isolated from young leaf material of the chrysanthemum *cv* Hero.

The chrysanthemum genomic DNA library was screened with ^{32}P-labelled fragments of a chrysanthemum *CHS* cDNA clone (contained in the plasmid pCGP856) using high

stringency conditions. The plasmid pCGP856 contains a 1.5 kb cDNA clone of *CHS* isolated from a petal cDNA library prepared from RNA isolated from the chrysanthemeum *cv.* Dark Pink Pom Pom.

A genomic clone (*CHS5*) was chosen for further analysis and found to contain ~3 kb of sequence upstream of the putative initiating methionine of the chrysanthemum *CHS* coding region.

A 4 kb fragment was released upon digestion of the genomic clone *CHS5* with the restriction endonuclease *Hin*dIII. The fragment containing the chrysanthemum *CHS* promoter was purified and ligated with *Hin*dIII ends of pBluescript SK (Stratagene, USA). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1316.

A 2.6 kb chrysanthemum *CHS* promoter fragment upstream from the putative translational initiation site was amplified by PCR using pCGP1316 as template and primers "chrysanCHSATG" (SEQ ID:8) and the M13 reverse primer (Stratagene, USA). Primer "chrysanCHSATG" incorporated an *Nco*I restriction endonuclease recognition sequence at the putative translation initiation point for ease of cloning. The PCR fragment was purified and ligated with *Eco*RV (dT-tailed) ends of pBluscript KS (Holton and Graham, *Nuc. Acids Res. 19:* 1156, 1990). Correct insertion of the fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1620.

<u>*Construction of pCGP1622 (chrysCHS 5': GUS: nos 3' in pUC backbone)*</u>

A ~2.5 kb fragment containing the chrysanthemum *CHS* promoter was released from the plasmid pCGP1620 upon digestion with the restriction endonucleases *Nco*I and *Pst*I. The fragmetn was purified and ligated with a 4.8 kb *NcoI/Pst*I fragment of pJB1 (Bodeau, 1994, *supra*) containing the backbone vector with the *GUS* and *nos 3'* fragments. Correct insertion of the fragment was established by restriction endonuclease analysis of DNA

isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1622.

Construction of pCGP1626 (chrysCHS 5': GUS: nos 3' in binary vector)

A ~4.6 kb fragment containing the *chrysCHS 5': GUS: nos 3'* cassette was released from the plasmid pCGP1622 upon digestion with the restriction endonucleases *Pst*I and *Bgl*II. The fragment was purified and ligated with *Pst*I/*Bam*HI ends of the Ti binary vector pWTT2132 (DNAP). Correct insertion of the cassette in a tandem orientation with respect to the *CaMV 35S: SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated as pCGP1626.

Plant transformation with pCGP1626

The T-DNA contained in the binary vector plasmid pCGP1626 was introduced into rose *via Agrobacterium*-mediated transformation.

Construction of pCGP1641 (petRT 5': GUS: petRT 3' binary vector)

The plasmid pCGP1641 contains a chimeric *GUS* gene under the control of a petunia *3RT* promoter (*petRT 5'*) covering 1.1kb upstream from the putative *3RT* translation initiation codon with a petunia *3RT* terminator (*petRT 3'*) covering 2.5 kb downstream from the 3RT stop codon. The chimeric *GUS* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassetteof the Ti binary vector, pWTT2132 (DNAP).

Isolation of petunia 3RT gene

The isolation of the petunia *3RT* gene corresponding to the *Rt* locus of *P. hybrida* has been described in Brugliera, 1994, *supra*.

Construction of pCGP1625 (CaMV 35S: GUS: petRT 3' cassette)

The intermediate plasmid pCGP1625 contains a *CaMV 35S: GUS: petRT 3'* cassette in a pUC backbone. The 2.5 kb fragment containing a petRT terminator sequences was

released from the plasmid pCGP1610 (described in Brugliera, 1994, *supra*) upon digestion with the restriction endonucleases *Bam*HI and *Sac*I. The fragment was purified and ligated with the *Bgl*II/*Sac*I 4.9kb fragment of pJB1 (Bodeau, 1994, *supra*) containing the vector backbone and the *CaMV 35S* promoter and *GUS* fragments. Correct insertion of the petunia *3RT* terminator fragment downstream of the *GUS* fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1625.

Construction of pCGP1628 (petRT 5': GUS: petRT 3' cassette)

A 1.1 kb *petRT* promoter fragment was released from the plasmid pCGP1611 (described in Brugliera, 1994, *supra*) upon digestion with the restriction endonucleases *Nco*I and *Pst*I. The purified fragment was ligated with *Nco*I/*Pst*I ends of the 7kb fragment of pCGP1625 containing the vector backbone and the *GUS* and *petRT 3'* fragments. Correct insertion of the *petRT* promoter fragment upstream of the *GUS* fragment was established by restriction endonuclease analysis of DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP1628.

Construction of pCGP1641 (petRT 5': GUS: petRT 3' binary vector)

A 5.4 kb fragment containing the *petRT 5': GUS: petRT 3'* cassette was released from pCGP1628 upon digestion with the restriction endonuclease *Pst*I. The fragment was purified and ligated with *Pst*I ends of the binary vector pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The resulting plasmid was designated as pCGP1641.

Plant transformation with pCGP1641

The T-DNA contained in the binary vector plasmid pCGP1641 was introduced into rose *via Agrobacterium*-mediated transformation.

Construction of pCGP1861 (RoseCHS 5': GUS: nos 3' binary vector)

The plasmid pCGP1861 contains a chimeric *GUS* gene under the control of a promoter fragment from the *CHS* gene of *R. hybrida* (*RoseCHS 5'*) with a terminator fragment from the *nos* gene of *Agrobacterium* (*nos 3'*). The chimeric *GUS* reporter gene cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassetteof the Ti binary vector, pWTT2132.

An ~5 kb fragment containing the *RoseCHS 5': GUS: nos 3'* cassette was released from pCGP197 (described in Example 4) upon digestion with the restriction endonuclease *Bgl*II. The fragment was purified and ligated with *Bam*HI ends of the Ti binary vector, pWTT2132 (DNAP). Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The resulting plasmid was designated as pCGP1861.

Plant transformation with pCGP1861

The T-DNA contained in the binary vector plasmid pCGP1861 was introduced into rose *via Agrobacterium*-mediated transformation.

Construction of pCGP1953 (AmCHS 5': GUS: petD8 3' binary vector)

The plasmid pCGP1953 contains a chimeric *GUS* gene under the control of a promoter fragment from the *CHS* gene of *Antirrhinum majus* (*AmCHS 5'*) with a petunia *PLTP* terminator (*petD8 3'*). The chimeric *GUS* reproter gene cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette of the Ti binary vector, pWTT2132 (DNAP).

The plasmid pJB1 (Bodeau, 1994, *supra*) was linearised with the restriction endonuclease *Nco*I. The overhanging ends were repaired and the 1.8 kb *GUS* fragment was released upon digestion with *Bam*HI. The *GUS* fragment was purified and was ligated with the 5 kb *Xba*I(ends repaired)/*Bam*HI fragment of pCGP726 containing the pBluescript backbone vector and the *AmCHS 5'* and *petD8 3'* fragments (described in Example 4). Correct

insertion of the *GUS* fragment between the *AmCHS 5'* and *petD8 3'* fragments was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The plasmid was designated as pCGP1952.

A 3.8 kb fragment containing the *AmCHS 5': GUS: petD8 3'* expression cassette was released from the plasmid pCGP1952 upon digestion with the restriction endonucleases *Eag*I and *Pst*I. The overhanging ends were repaired and the purified fragment was ligated with the repaired ends of an *Asp*718 digested pWTT2312 Ti binary vector. Correct insertion of the fragment in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassette was established by restriction endonuclease analysis of plasmid DNA isolated from tetracyline-resistant transformants. The plasmid was designated as pCGP1953.

Plant transformation with pCGP1953

The T-DNA contained in the binary vector plasmid pCGP1953 was introduced into rose *via Agrobacterium*-mediated transformation.

Construction of pWTT2084 (CaMV 35S: GUS: ocs 3' binary vector)

The plasmid pWTT2084 (DNAP) contains a chimeric *GUS* gene under the control of a *CaMV 35S* promoter (*CaMV 35S*) with an octopine synthase terminator (*ocs 3'*). An ~60 bp 5' untranslated leader sequence from the petunia chlorophyll a/b binding protein gene (*Cab 22* gene) (Harpster *et al.*, 1988, *supra*) is included between the *CaMV 35S* promoter fragment and the *GUS* clone. The chimeric *GUS* cassette is in a tandem orientation with respect to the *CaMV 35S*: *SuRB* selectable marker gene cassetteof the Ti binary vector, pWTT2132.

Plant transformation with pWTT2084

The T-DNA contained in the binary vector plasmid pWTT2084 was introduced into rose *via Agrobacterium*-mediated transformation.

Transgenic analysisof roses transfromed with GUS expression cassettes

Northern analysis was performed on RNA isolated from petals of developmental stages 3 and 4 of transgenic Kardinal roses transformed with the T-DNA of various *GUS* expression cassettes. The relative levels of *GUS* transcripts accumulating in the rose petals were recorded (see Table 7).

TABLE 7 Summary of Northern analysis on transgenic Kardinal rose flowers (open bud stage) containing *GUS* constructs.

Construct number	*GUS* reporter gene cassette	Selectable marker gene cassette	*GUS* transcript levels
pCGP1307	*petD8 5': GUS: petD8 3'*	*mas 5': nptII :mas 3'*	–
pCGP1506	*petFLS 5': GUS: petFLS 3'*	*nos 5': nptII: nos 3'*	–
pCGP1626	*chrysCHS 5': GUS: petRT 3'*	*CaMV 35S: SuRB*	++ to +++
pCGP1641	*petRT 5': GUS: petRT 3'*	*CaMV 35S: SuRB*	–
pCGP1861	*RoseCHS 5': GUS: nos 3'*	*CaMV 35S: SuRB*	++++
pCGP1953	*AmCHS 5': GUS: petD8 3'*	*CaMV 35S: SuRB*	–
pWTT2084	*CaMV 35S: GUS: ocs 3'*	*CaMV 35S: SuRB*	+++++

– = no transcripts detected.

+ to +++++ = very low to very high levels of transcript detected.

Based on the above results (Table 7), the *CaMV 35S* and Rose *CHS* promoters appear to promote relatively high levels of transcription in rose petals. The chrysanthemum *CHS* promoter appears to also lead to high transcript levels but not as high as those achieved using *CaMV 35S* or Rose *CHS* promoters. Surprisingly, antirrhinum (snapdragon) *CHS*, petunia *3RT*, petunia *FLS* and petunia *PLTP* (D8) promoters did not appear to function in rose petals with no detectable *GUS* transcripts accumulating using expression cassettes incorporating these promoters. These promoters had previously been proven to function well in carnation and petunia. The result obtained with the antirrhinum *CHS* promoter linked to *GUS* was more surprising as the *CHS* promoters from two other species (rose and chrysanthemum) appeared to function relatively well in roses. The antirrhinum *CHS*

promoter had also been successfully used in conjunction with petunia F3'5'H (*Hf1*) to produce the novel violet colored-carnations Florigene Moondust (see International Patent Application No. PCT/AU96/00296).

These results also provided further evidence to suggest that the petunia *Hf1* and *Hf2* sequences were unstable in roses as constructs containing these sequences ligated to the *CaMV 35S*, *Mac*, rose *CHS* and chrysanthemum *CHS* promoters did not lead to intact *Hf1* or *Hf2* transcripts in roses.

Analysis of the petunia *F3'5'H* nucleotide sequences (*Hf1* and *Hf2*) did not reveal any instability sequences (Johnson *et al.*, *In A look beyond transcription, ASPP*, USA, Bailey-Serres and Gallie, eds, 1998), intron: exon splice junctions (Brendel *et al.*, *In A look beyond transcription, ASPP*, USA, Bailey-Serres and Gallie, eds, 1998), or any autocatalytic or degradation trigger sequences reported in the scientific literature to date (*In A look beyond transcription, ASPP*, USA, Bailey-Serres and Gallie, eds, 1998).

Since it was not obvious why the petunia *F3'5'H* sequences were unstable in roses but stable in carnation, petunia or tobacco a number of *F3'5'H* sequences were isolated across a range of families in an attempt to demonstrate delphinidin production in roses through synthesis of stable *F3'5'H* transcripts and F3'5'H activity.

EXAMPLE 8

Isolation of F3'5'H sequences from species other than petunia

Construction of petal cDNA libraries

Petal cDNA libraries were prepared from RNA isolated from petals from bud to opened flower stages from various species of plants described in Table 8. *Rosa hybrida* is classified in the family Rosaciae, Order Rosales, Subclass Rosidae and so species that produced delphinidin-based pigments and so contained a functional F3'5'H and belonged to the Subclass Rosidae were selected. *Petunia hybrida* is classified in the Family

Solanaceae, Order Solanales, Subclass Asteridae and so species from the Subclass Asteridae that produced delphinidin-based pigments were also selected.

TABLE 8 List of flowers from which cDNA libraries were prepared.

Flower	*Species*	Family	Order	Subclass
gentian	*Gentiana spp.*	Gentianaceae	Gentianales	Asteridae
pansy	*Viola spp.*	Violaceae	Malpighiales	Rosidae
salvia	*Salvia spp.*	Labiatae	Lamiales	Asteridae
sollya	*Sollya spp.*	Pittosporaceae	Apiales	Asteridae
kennedia	*Kennedia spp.*	Leguminosae	Fabales	Rosidae
butterfly pea	*Clitoria ternatea*	Leguminosae	Fabales	Rosidae

Information obtained from (National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) under Taxonomy browser (TaxBrowser) (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/)).

Unless otherwise described, total RNA was isolated from the petal tissue of purple/blue flowers using the method of Turpen and Griffith (*BioTechniques 4:* 11-15, 1986). Poly(A)$^+$ RNA was selected from the total RNA, using oligotex-dT^{TM} (Qiagen) or by three cycles of oligo-dT cellulose chromatography (Aviv and Leder, *Proc. Natl. Acad. Sci. USA 69*: 1408, 1972).

In general a λZAPII/ Gigapack II Cloning kit (Stratagene, USA) (Short *et al.*, *Nucl. Acids Res. 16:* 7583-7600, 1988) was used to construct directional petal cDNA libraries in λZAPII using around 5 μg of poly(A)$^+$ RNA isolated from petal as template. The total number of recombinants obtained was generally in the order of 1 x 10^5 to 1 x 10^6.

After transfecting XL1-Blue MRF' cells, the packaged cDNA mixtures were plated at around 50,000 pfu per 15 cm diameter plate. The plates were incubated at 37°C for 8 hours, and the phage were eluted in 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH

8.0, 0.01% (w/v) gelatin (Phage Storage Buffer (PSB)) (Sambrook *et al.*, 1989, *supra*). Chloroform was added and the phages stored at 4°C as amplified libraries.

In general around 100,000 pfu of the amplified libraries were plated onto NZY plates (Sambrook *et al.*, 1989, *supra*) at a density of around 10,000 pfu per 15 cm plate after transfecting XL1-Blue MRF' cells, and incubated at 37°C for 8 hours. After incubation at 4°C overnight, duplicate lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Plasmid Isolation

Helper phage R408 (Stratagene, USA) was used to excise pBluescript phagemids containing cDNA inserts from amplified λZAPII or λZAP cDNA libraries using methods described by the manufacturer.

Screening of petal cDNA Libraries

Prior to hybridization, duplicate plaque lifts were washed in prewashing solution (50 mM Tris-HCl pH7.5, 1 M NaCl, 1 mM EDTA, 0.1% (w/v) sarcosine) at 65°C for 30 minutes; followed by washing in 0.4 M sodium hydroxide at 65°C for 30 minutes; then washed in a solution of 0.2 M Tris-HCl pH 8.0, 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 30 minutes and finally rinsed in 2 x SSC, 1.0% (w/v) SDS.

The membrane lifts from the petal cDNA libraries were hybridized with ^{32}P-labelled fragments of a 1.6 kb *Bsp*HI/*Fsp*I fragment from pCGP602 containing the petunia *F3'5'H Hf1* cDNA clone (Holton *et al.*, 1993, *supra*).

Hybridization conditions included a prehybridization step in 10% v/v formamide, 1 M NaCl, 10% w/v dextran sulphate, 1% w/v SDS at 42°C for at least 1 hour. The ^{32}P-labelled fragments (each at $1x10^6$ cpm/mL) were then added to the hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed in 2 x SSC, 1% w/v SDS at 42°C for 2 x 1 hour and exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

Strongly hybridizing plaques were picked into PSB (Sambrook *et al.*, 1989, *supra*) and rescreened to isolate purified plaques, using the plating and hybridization conditions as described for the initial screening of the cDNA library. The plasmids contained in the λZAPII or λZAP bacteriophage vectors were rescued and sequence data was generated from the 3' and 5' ends of the cDNA inserts. New *F3'5'H* cDNA clones were identified based on sequence similarity to the petunia *Hf1* cDNA clone.

The cDNA clones isolated were given plasmid designation numbers as described in Table 9.

TABLE 9 Plasmid numbers and SEQ ID NO. of *F3'5'H* cDNA clones isolated from various species

Species	Clone	Plasmid number	SEQ ID NO.
Viola spp.	BP#18	pCGP1959	9
Viola spp.	BP#40	pCGP1961	11
Salvia spp.	Sal#2	pCGP1995	13
Salvia spp.	Sal#47	pCGP1999	15
Sollya spp.	Soll#5	pCGP2110	17
Kennedia	Kenn#31	pCGP2231	-
Butterfly Pea	BpeaHF2	pBHF2 or 4	20
Gentian	Gen#48	pG48	22

Isolation of F3'5'H cDNA clones from petals of Viola spp.

Total RNA and poly $(A)^+$ RNA was isolated from petals of young buds of *Viola spp.* cultivar black pansy as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA) and screened as described above. Two full-length pansy *F3'5'H* cDNA clones (*BP#18* (SEQ ID NO:9) in pCGP1959 and BP#40 (SEQ ID NO:11) in pCGP1961) were identified by sequence similarity to the petunia *Hf1* cDNA clone (SEQ ID NO:1). The BP#18 and BP#40 shared 82% identity at the nucleotide level. Comparison of the nucleotide sequence of pansy *F3'5'H* clones (BP#18 and BP#40) with that of the petunia *F3'5'H* revealed around 60% identity to the petunia *Hf1* clone and 62% identity to the petunia *Hf2* clone.

Construction of binary vectors, pCGP1972 and pCGP1973

(*AmCHS 5'*: pansy*F3'5'H* #18 or #40: *petD8 3'*)

The plasmids pCGP1972 and pCGP1973 contain the pansy *F3'5'H* cDNA clone (BP#18 and BP#40, respectively) between an *A. majus* (snapdragon) *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*). The chimeric *F3'5'H* genes are in tandem with respect to the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector, pWTT2132 (DNAP).

The petunia *F3'5'H* (*Hf1*) cDNA clone in pCGP725 was replaced with the pansy *F3'5'H* BP#18 or BP#40 cDNA clone to produce pCGP1970 and pCGP1971, respectively. The *AmCHS 5': pansy F3'5'H: petD8 3'* cassette was then isolated from pCGP1970 or pCGP1971 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearised plasmid were repaired and then the chimeric *F3'5'H* genes were released upon digestion with the restriction endonuclease *Eco*RV. The purified fragments were then ligated with *Asp*718 repaired ends of the Ti binary vector pWTT2132 (DNAP). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP1972 and pCGP1973, respectively.

Carnation and petunia transformation with pCGP1972 and 1973

The T-DNAs contained in the binary vector plasmids pCGP1972 and pCGP1973 were introduced separately into *Dianthus caryoplhyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida cv.* Skr4 x Sw63 via *Agrobacterium*-mediated transformation.

Construction of binary vectors, pCGP1967 and pCGP1969

(CaMV 35S: pansy F3'5'H: ocs 3')

The binary vectors pCGP1967 and pCGP1969 contain chimeric *CaMV 35S: pansy F3'5'H: ocs 3'* genes in tandem with respect to the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector, pWTT2132 (DNAP).

The plasmids pCGP1959 and pCGP1961 were firstly linearized upon digestion with the restriction endonuclease *Kpn*I. The overhanging *Kpn*I ends were repaired and the pansy *F3'5'H* cDNA clones, BP#18 and BP#40, were released upon digestion with the restriction endonuclease *Pst*I. The ~1.7 kb fragments generated were ligated with an ~5.9 kb *Eco*RI (repaired ends)/*Pst*I fragment of pKIWI101 (Klee *et al.*, 1985, *supra)*. Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmids were designated pCGP1965 and pCGP1966, respectively.

The plasmids pCGP1965 and pCGP1966 were firstly partially digested with the restriction endonuclease *Xho*I. The resulting overhanging 5' ends were repaired and then the fragments were further digested with the restriction endonuclease *Xba*I. The 3.6kb fragments containing the *CaMV 35S: pansy F3'5'H: ocs 3'* chimeric genes were isolated and ligated with *Asp*718 repaired ends of pWTT2132. Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP1967 and pCGP1969, respectively.

Rose transformation

The T-DNAs contained in the binary vector plasmids pCGP1967 and pCGP1969 were introduced separately into *Rosa hybrida cv.* Kardinal and Soft Promise via *Agrobacterium*-mediated transformation. The T-DNA contained in the binary vector plasmids pCGP1969 was also introduced into *Rosa hybrida cv.* Pamela and Medeo via *Agrobacterium*-mediated transformation.

Isolation of a F3'5'H cDNA clone from petals of Salvia spp.

Total RNA and poly $(A)^+$ RNA was isolated from young petal buds of *Salvia spp.* (bought from a nursery) as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA). Two full-length salvia *F3'5'H* cDNA clones (Sal#2 (SEQ ID NO:13) in pCGP1995 and Sal#47 (SEQ ID NO:15) in pCGP1999) were identified by sequence similarity with the petunia *Hf1* cDNA clone. The Sal#2 and Sal#47

shared 95% identity at the nucleotide level. Comparison of the nucleotide sequence of salvia *F3'5'H* clones (Sal#2 and Sal#47) with that of the petunia *F3'5'H* revealed around 57% identity to the petunia *Hf1* clone and 58% identity to the petunia *Hf2* clone.

Construction of binary vectors, pCGP2121 and pCGP2122

(AmCHS 5': Salvia F3'5'H #2 or #47: petD8 3')

The plasmids pCGP2121 and pCGP2122 contain the salvia *F3'5'H* cDNA clones (Sal#2 and Sal#47, respectively) between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pWTT2132 (DNAP).

The petunia *F3'5'H* (*Hf1*) cDNA clone in pCGP725 (described in Example 4) was replaced with the salvia *F3'5'H* #2 or #47 cDNA clones to produce pCGP2116 and pCGP2117, respectively. The *AmCHS 5': salvia F3'5'H: petD8 3'* cassette was then isolated from pCGP2116 or pCGP2117 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearized plasmid were repaired and then the chimeric *F3'5'H* gene cassettes were released upon digestion with the restriction endonuclease *Eco*RV. The purified fragments were then ligated with *Asp*718 repaired ends of the Ti binary vector pCGP1988 (described in Example 4). Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP2121 and pCGP2122, respectively.

Carnation and petunia transformation with pCGP2121 and pCGP2122

The T-DNAs contained in the binary vector plasmids pCGP2121 and pCGP2122 were introduced separately into *Dianthus caryoplhyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida cv.* Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

Construction of binary vectors, pCGP2120 and pCGP2119

(CaMV 35S: salvia F3'5'H: ocs 3')

The binary vectors pCGP2119 and pCGP2120 contain chimeric *CaMV 35S: salvia F3'5'H: ocs 3'* gene cassettes in tandem with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pCGP1988.

The plasmids pCGP1995 and pCGP1999 were firstly linearized upon digestion with the restriction endonuclease *Xho*I. The overhanging *Xho*I ends were repaired and then the salvia *F3'5'H* cDNA clones Sal#2 or Sal#47 were released upon digestion with the restriction endonuclease *Eco*RI. In the case of pCGP1995 a partial digest with *Eco*RI was undertaken. The ~1.7 kb fragments were ligated with the *Cla*I (repaired ends)/*Eco*RI ends of pCGP2105. Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmids were designated pCGP2112 and pCGP2111, respectively.

The plasmids pCGP2112 and pCGP2111 were firstly linearized with the restriction endonuclease *Xho*I. The resulting overhanging 5' ends were repaired and then the fragments were further digested with the restriction endonuclease *Xba*I. The 3.6 kb fragments containing the *CaMV 35S: salvia F3'5'H: ocs 3'* chimeric genes were isolated and ligated with *Asp*718 repaired ends of the Ti binary vector, pCGP1988 (described in Example 4). Correct insertion of each fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmids were designated pCGP2120 and pCGP2119, respectively.

Rose transformation with pCGP2120 and pCGP2119

The T-DNAs contained in the binary vector plasmids pCGP2120 and pCGP2119 were introduced separately into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Isolation of a F3'5'H cDNA clone from petals of Sollya spp.

Total RNA and poly $(A)^+$ RNA was isolated from young petal buds of *Sollya spp.* (bought from a nursery) as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA). One full-length Sollya *F3'5'H* cDNA clone (Soll#5 (SEQ ID NO:17) in pCGP2110) was identified by sequence similarity to the petunia *Hf1* cDNA clone. Comparison of the nucleotide sequence of sollya *F3'5'H* clones with that of the petunia *F3'5'H* revealed around 48% identity to the petunia *Hf1* clone and 52% identity to the petunia *Hf2* clone.

Construction of binary vector, pCGP2130 (AmCHS 5': Sollya F3'5'H: petD8 3')

The plasmid pCGP2121 contains the sollya *F3'5'H* Soll#5 cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in a tandem orientation with respect to the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pCGP1988.

The petunia *F3'5'H* (*Hf1*) cDNA clone in pCGP725 (described in Example 4) was replaced with the sollya *F3'5'H* cDNA clone to produce pCGP2128. The *AmCHS 5':* sollya *F3'5'H: petD8 3'* gene cassette was then isolated from pCGP2128 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearized plasmid were repaired and then the chimeric *F3'5'H* gene was released upon digestion with the restriction endonuclease *Eco*RV. The purified fragment was then ligated with *Asp*718 (repaired ends) of the Ti binary vector pCGP1988 (described in Example 4). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2130.

Carnation and petunia transformation with pCGP2130

The T-DNA contained in the binary vector plasmid pCGP2130 was introduced into *Dianthus caryoplhyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida cv.* Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

Construction of binary vectors, pCGP2131 (CaMV 35S: sollya F3'5'H: ocs 3')

The binary vector pCGP2131 contains a chimeric *CaMV 35S: sollya F3'5'H: ocs 3'* gene in tandem with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pCGP1988.

The plasmid pCGP2110 was firstly linearized upon digestion with the restriction endonuclease *Asp*718. The overhanging ends were repaired and then the sollya *F3'5'H* cDNA clone was released upon digestion with the restriction endonuclease *Pst*I. The ~1.7 kb fragment was ligated with the *Eco*RV/*Pst*I ends of pCGP2105. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP2129.

A 3.6 kb fragment containing the *CaMV 35S: sollya F3'5'H: ocs 3'* chimeric gene was released upon digestion with the restriction endonucleases *Asp*718 and *Xba*I The overhanging ends were repaired and the purified fragment was ligated with of *Asp*718 repaired ends of the Ti binary vector, pCGP1988. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2131.

Rose transformation with pCGP2131

The T-DNA contained in the binary vector plasmid pCGP2131 was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Isolation of a F3'5'H cDNA clone from petals of Kennedia spp.

Total RNA and poly $(A)^+$ RNA was isolated from young petal buds of *Kennedia spp.* (bought from a nursery) as described above. A petal cDNA library was constructed using λZAPII/ Gigapack II Cloning kit (Stratagene, USA). One full-length kennedia *F3'5'H* cDNA clone (*Kenn#31* in pCGP2231) was identified by sequence similarity to the petunia *Hf1* cDNA clone.

Construction of binary vector, pCGP2256 (AmCHS 5': kennedia F3'5'H: petD8 3')

The plasmid pCGP2156 contains the kennedia *F3'5'H* (*Kenn#31*) cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pCGP1988.

The petunia *F3'5'H* (*Hf1*) cDNA clone in pCGP725 (described in Example 4) was replaced with the kennedia *F3'5'H* (*Kenn#31*) cDNA clone to produce pCGP2242. The *AmCHS 5': kennedia F3'5'H: petD8 3'* cassette was then isolated from pCGP2242 by digesting with the restriction endonucleases *Not*I and *Eco*RI. The ends were repaired and the purified fragment was then ligated with *Asp*718 repaired ends of the Ti binary vector pCGP1988 (described in Example 4). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2256.

Petunia transformation with pCGP2256

The T-DNA contained in the binary vector plasmid pCGP2256 was introduced into *Petunia hybrida* cv. Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

Construction of binary vectors, pCGP2252

(CaMV 35S: kennedia F3'5'H: ocs 3')

The binary vector pCGP2252 contains a chimeric *CaMV 35S: kennedia F3'5'H: ocs 3'* gene in tandem with the *CaMV 35S: SuRB* selectable marker cassette of the Ti binary vector pCGP1988.

The plasmid pCGP2231 was firstly linearized upon digestion with the restriction endonuclease *Xho*I. The overhanging ends were repaired and then the kennedia *F3'5'H* cDNA clone was released upon digestion with the restriction endonuclease *Pst*I. The ~1.7 kb fragment was ligated with the *Cla*I (repaired ends)/*Pst*I ends of pCGP2105. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid

DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP2236.

A 3.6 kb fragment containing the *CaMV 35S: kennedia F3'5'H: ocs 3'* chimeric gene cassette was released from the plasmid pCGP2236 upon digestion with the restriction endonucleases *Xho*I and *Not*I. The overhanging ends were repaired and the purified fragment was ligated with *Asp*718 repaired ends of the Ti binary vector, pCGP1988. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2252.

Rose transformation with pCGP2252

The T-DNA contained in the binary vector plasmid pCGP2252 was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Isolation of a F3'5'H cDNA clone from petals of Clitoria ternatea (butterfly pea)

Construction of butterfly pea petal cDNA library

A blue variety of *Clitoria ternatea* (butterfly pea, the seeds were kindly provided by Osaka Botanical Garden) was grown in a field in Osaka. Total RNA of fresh and pigmented petals at a pre-anthesis stage was prepared as mentioned above. PolyA$^+$ RNA was prepared using Oligotex (Takara) according to the manufacturer's recommendation. A petal cDNA library of butterfly pea was constructed from the polyA$^+$ RNA using a directional λZAP-cDNA synthesis kit (Stratagene, USA) following the manufacturer's protocols.

Screening of butterfly pea cDNA library for a F3'5'H cDNA clone

The butterfly pea petal cDNA library was screened with DIG-labelled petunia *Hf1* cDNA clone as described previously (Tanaka *et al., Plant Cell Physiol. 37:* 711-716, 1996). Two cDNA clones that showed high similarity to *Hf1* were identified. The plasmid containing the longest cDNA clone was designated pBHF2 and the cDNA clone was sequenced (SEQ ID NO:20). Alignment between the deduced amino acid sequences of the butterfly pea *F3'5'H* clone and the petunia *Hf1* clone revealed that the butterfly pea *F3'5'H* cDNA

(contained in pBHF2) did not represent a full-length cDNA and lacked first 2 bases of the putative initiation codon. These two bases along with a *Bam*HI restriction endonuclease recognition site were added to the cDNA clone using PCR and a synthetic primer, 5'-GGGATCCAACAATGTTCCTTCTAAGAGAAAT-3' [SEQ ID NO:25] as described previously (Yonekura-Sakakibara *et al., Plant Cell Physiol. 41:* 495-502, 2000). The resultant fragment was digested with the restriction endonucleases *Bam*HI and *Pst*I and the subsequent DNA fragment of about 200 bp was recovered. The DNA fragment was ligated with a 3.3 kb fragment of *Bam*HI/*Eco*RI digested pBHF2 to yield pBHF2F. The DNA sequence was confirmed to exclude errors made during PCR.

Comparison of the nucleotide sequence of butterfly pea *F3'5'H* clone with that of the petunia *F3'5'H* revealed around 59% identity to the petunia *Hf1* clone and 62% identity to the petunia *Hf2* clone.

Construction of binary vector, pCGP2135 (AmCHS 5': butterfly pea F3'5'H: petD8 3')

The plasmid pCGP2156 contains the butterfly pea *F3'5'H* cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pCGP1988.

The petunia *F3'5'H* (*Hf1*) cDNA clone in pCGP725 (described in Example 4) was replaced with the butterfly pea *F3'5'H* cDNA clone to produce pCGP2133. The *AmCHS 5': butterfly pea F3'5'H: petD8 3'* cassette was then isolated from pCGP2133 by firstly digesting with the restriction endonuclease *Not*I. The ends of the linearised plasmid were repaired and then the chimeric *F3'5'H* gene was released upon digestion with the restriction endonuclease *Eco*RV. The purified fragment was then ligated with *Asp*718 repaired ends of the Ti binary vector pCGP1988 (described in Example 4). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2135.

Carnation and petunia transformation with pCGP2135

The T-DNA contained in the binary vector plasmid pCGP2135 was introduced into *Dianthus caryoplhyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida cv.* Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

Construction of the binary vector, pBEBHF2 (CaMV 35S: Butterfly pea F3'5'H: nos 3')

The Ti binary vector, pBE2113-GUS contains a *GUS* coding region between an enhanced *CaMV 35S* promoter and *nos* terminator (Mitsuhara *et al., Plant Cell Physiol. 37:* 49-59, 1996). The plasmid pBE2113-GUS was digested with the restriction endonuclease *Sac*I. The overhanging ends were repaired and then ligated with a *Sal*I linker to yield pBE2113-GUSs. The 1.8 kb *Bam*HI-*Xho*I fragment from pBHF2F was ligated with *Bam*HI-*Sal*I digested pBE2113-GUSs to create pBEBHF2.

Rose transformation with pBEBHF

The T-DNA contained in the binary vector plasmid pBEBHF was introduced into *Rosa hybrida* cultivar Lavande *via Agrobacterium*-mediated transformation.

Construction of binary vectors, pCGP2134 (CaMV 35S: butterfly pea F3'5'H: ocs 3')

The binary vector pCGP2134 contains a chimeric *CaMV 35S: butterfly pea F3'5'H: ocs 3'* gene cassette in a tandem orientation with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pCGP1988.

The butterfly pea *F3'5'H* cDNA clone was released upon digestion of the plasmid pBHF53 with the restriction endonucleases *Xho*I and *Bam*HI. The overhanging ends were repaired and the ~1.7 kb fragment was ligated with the *Pst*I (repaired ends)/*Eco*RV ends of pCGP2105 (described in Example 4). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP2132.

A 3.6 kb fragment containing the *CaMV 35S: butterfly pea F3'5'H: ocs 3'* chimeric gene cassette was released upon digestion with the restriction endonucleases *Xho*I and *Xba*I. The

overhanging ends were repaired and the purified fragment was ligated with *Asp*718 repaired ends of the Ti binary vector, pCGP1988 (described in Example 4). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2134.

Rose transformation with pCGP2134

The T-DNA contained in the binary vector plasmid pCGP2134 was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

Isolation of a F3'5'H cDNA clone from petals of Gentiana triflora (gentian).

Construction and screening of a gentian petal cDNA library

The isolation of a gentian cDNA encoding *F3'5'H* has been described previously (Tanaka *et al.*, 1996, *supra*). Comparison of the nucleotide sequence of the gentian *F3'5'H* clone (*Gen#48*) (SEQ ID NO:22) with that of the petunia *F3'5'H* revealed around 61% identity to the petunia *Hf1* clone and 64% identity to the petunia *Hf2* clone.

Construction of binary vector, pCGP1498 (AmCHS 5': gentian F3'5'H: petD8 3')

The plasmid pCGP2121 contains the gentian *F3'5'H* (*Gen#48*) cDNA clone between a snapdragon *CHS* promoter fragment (*AmCHS 5'*) and a petunia *PLTP* terminator fragment (*petD8 3'*) in tandem with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pWTT2132.

The petunia *F3'5'H* (*Hf1*) cDNA clone in pCGP725 (described in Example 4) was replaced with the gentian *F3'5'H* (*Gen#48*) cDNA clone to produce pCGP1496. The *AmCHS 5'*: gentian *F3'5'H*: *petD8 3'* cassette was then isolated from pCGP1496 by firstly digesting with the restriction endonuclease *Not*I. The overhanging ends of the linearised plasmid were repaired and then the chimeric *F3'5'H* gene was released upon digestion with the restriction endonuclease *Eco*RV. The purified fragment was then ligated with *Asp*718 repaired ends of the Ti binary vector pWTT2132. Correct insertion of the fragment was

established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP1498.

Carnation and petunia transformation with pCGP1498

The T-DNA contained in the binary vector plasmid pCGP1498 was introduced into *Dianthus caryoplhyllus* cultivars Kortina Chanel and Monte Lisa and *Petunia hybrida* cv. Skr4 x Sw63 *via Agrobacterium*-mediated transformation.

Construction of the binary vector, pBEGHF48 (CaMV 35S: gentian F3'5'H: nos 3')

The gentian *F3'5'H* cDNA clone was released by digestion of the plasmid pG48 with the restriction endonucleases *Bam*HI and *Xho*I. The resulting 1.8 kb DNA fragment was isolated and ligated with *Bam*HI/*Sal*I digested pBE2113-GUSs (Mitsuhara *et al.*, 1996, *supra*) to create pBEGHF48.

Rose transformation with pBEGHF48

The T-DNA contained in the binary vector plasmid pBEGHF48 was introduced into *Rosa hybrida cv.* Lavande *via Agrobacterium*-mediated transformation.

Construction of binary vectors, pCGP1982 (CaMV 35S: gentian F3'5'H: ocs 3')

The binary vector pCGP1982 contains a chimeric *CaMV 35S: gentian F3'5'H: ocs 3'* gene cassette in tandem with the *CaMV 35S: SuRB* selectable marker gene cassette of the Ti binary vector pWTT2132.

The plasmid pG48 was firstly linearised upon digestion with the restriction endonuclease *Asp*718. The overhanging ends were repaired and then the gentian *F3'5'H* cDNA clone (*Gen#48*) was released upon digestion with the restriction endonuclease *Bam*HI. The ~1.7 kb fragment was ligated with the 5.95kb *Eco*RI (repaired ends)/*Bam*HI fragment of pKIWI101 (Klee *et al.*, 1985, *supra*). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP1981.

A 3.6 kb fragment containing the *CaMV 35S: gentian F3'5'H: ocs 3'* chimeric gene cassette was released upon digestion with the restriction endonucleases *Xho*I and *Xba*I The overhanging ends were repaired and the purified fragment was ligated with repaired ends of *Asp*718 digested Ti binary vector, pWTT2132. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP1982.

Rose transformation with pCGP1982

The T-DNA contained in the binary vector plasmid pCGP1982 was introduced into *Rosa hybrida cv.* Kardinal *via Agrobacterium*-mediated transformation.

EXAMPLE 9

Analysis of transgenic carnation, petunia and rose

Transgenic analysis of events transfromed with the T-DNA of binary vectors described in Example 9 included detection of F3'5'H activity via the presence of the 3'5'-hydroxylated anthocyanidin, delphinidin or in the case of petunia, its derivatives such as malvidin, and detection of intact transcripts of the introduced F3'5'H (see Tables 10, 11 and 12).

Carnation

TABLE 10 Results of levels of delphinidin produced in transgenic carnations using various *F3'5'H* gene expression cassettes (*AmCHS 5'*: *F3'5'H*: *petD8 3'*).

F3'5'H clone	pCGP	Cv.	#tg	TLC+	HPLC+	Highest % del	Av. % del	Northern+
Salvia#2	2121	KC	22	2/16	3/4	12.5%	7%	*nd*
	2121	ML	21	17/18	9/9	76%	57%	14/15
Salvia#47	2122	KC	23	6/12	8/8	29%	12%	*nd*
	2122	ML	25	21/22	17/17	88%	56%	12/14
Sollya	2130	KC	30	22/27	17/17	35%	11%	*nd*
	2130	ML	23	14/15	14/14	76%	49%	13/14
Butterfly pea	2135	KC	22	0/16	0/1	*nd*	*nd*	*nd*
	2135	ML	24	19/20	13/13	23%	10%	14/14

F3'5'H clone	pCGP	Cv.	#tg	TLC+	HPLC+	Highest % del	Av. % del	Northern+
Gentian	1498	KC	22	0/14	*nd*	*nd*	*nd*	7/8
	1498	ML	2	2/2	1/1			1/2
pansy BP#18	1972	KC	26	18/20	12/12	14%	9%	19/19
	1972	ML	21	15/16	8/8	80%	66%	14/16
pansy BP#40	1973	KC	26	11/15	7/8	18%	8%	13/17
	1973	ML	33	19/22	20/20	72%	52%	12/15
petunia Hf1	1452	KC	104	41/64		3.5%	1.3%	15/17
	1452	ML	48	39/41	26/26	75%	30%	12/13
petunia Hf2	1524	ML	27	18/19	17/17	81%	41%	12/14

Cv. = cultivar

KC = Kortina Chanel (cyanidin line)

ML = Monte Lisa (pelargonidin line)

#tg = # of transgenics produced

TLC+ = number of individual events that accumulated detectable delphinidin (as determined by TLC)/the number of individual events analyzed

HPLC+ = number of individual events that accumulated detectable delphinidin (as determined by HPLC) / the number of individual events analyzed

Highest % del = Highest % delphinidin recorded for the population.

Av % del = average % delphinidin detected in population.

Northern = number of individual events with detectable *F3'5H* transcripts/the number analyzed

Kortina Chanel produces pink colored flowers that normally accumulate cyanidin-based anthocyanins. This cultivar therefore contains a functional carnation F3'H and DFR activity that the introduced *F3'5'H* would need to compete with for substrate. Monte Lisa produces brick red colored flowers that normally accumulate pelargonidin. This cultivar is thought to lack a fully functional F3'H activity and contain a DFR that is capable of acting on DHK and thus the introduced *F3'5'H* would only be required to compete with the endogenous DFR for substrate.

The results suggest that all of the *F3'5'H* sequences tested (petunia *Hf1*, petunia *Hf2*, Salvia *Sal#2*, Salvia *Sal#47*, Sollya *Sol#5*, Butterfly pea *BpeaHF2*, pansy *BP#18*, pansy *BP#40* and Gentian *Gen#48*) were functional in carnation and resulted in the production of novel delphinidin-based pigments in carnation flowers.

Petunia

TABLE 11 Results of analysis of transgenic *P. hybrida* cv Skr4 x Sw63 using various *F3'5'H* gene expression cassettes (*AmCHS 5'*: *F3'5'H*: *petD8 3'*).

F3'5'H	pCGP	# tg	TLC+	Col	↑ A/c	Best	Av.	Northern+	Best color
Gentian	1498	22	3/5	18/20	*nd*			6/6	72B/78A
Butterfly pea	2135	24	18/20	22/24	23/24	4427	2397		74A/78A
Kennedia	2256	24	22/24	22/24	22/24	4212	2592	*nd*	74A/78A
Salvia2	2121	24	21/24	21/24	21/24	2471	1730		78A
Salvia47	2122	19	17/19	16/19	16/19	2634	1755		78A/80A
Sollya	2130	22	14/16	13/16	13/16	3446	1565		78A
pansy 18	1972	22	*nd*	20/22	*nd*			9/9	74A/B
pansy 40	1973	19	8/8	18/19	18/20	2583	1556		74/78A
petunia Hf1	484	16	*nd*	9/16	8/15	2683	1250		74A/B
petunia Hf2	1524	20	*nd*	18/20	8/8	4578	2357	8/8	74A/B
control						144-250			75C

#tg = # of transgenics produced

TLC+ = number of individual events that accumulated detectable malvidin (above the Skr4 x Sw63 background) (as determined by TLC)/the number of individual events analyzed

Col = number of individual events that had a change in phenotype/number examined

↑ A/c = number of individual events that had an increased level of anthocyanins as measured by spectrophotometric analysis/the number of individual events analyzed (in μmoles/g)

Best = the highest anthocyanin amount found in an individual event (in μmoles/g)

Av = the average anthocyaninin levels detected (in μmoles/g).

Northern = number of individual events with detectable *F3'5H* transcripts over the number analyzed

Best color = most intense color recorded for the transgenic population.

Introduction of the *F3'5'H* cDNA clones into Skr4 x SW63 led to a dramatic flower color change from pale lilac to purple and to the production of malvidin in the petals. Malvidin is the methylated derivative of the 3'5'-hydroxylated pigment, delphinidin (Figures 1A and 1B). Only a small amount of malvidin is normally detected in the non-transgenic Skr4 x SW63 control. Although Skr4 x SW63 is homozygous recessive for both the *Hf1* and *Hf2* genes, these mutations do not completely block production of *F3'5'H* (see US Patent Number 5,349,125) and low levels of malvidin are produced to give the petal limb a pale lilac color.

The results suggest that all of the *F3'5'H* sequences tested (petunia *Hf1*, petunia *Hf2*, Salvia *Sal#2*, Salvia *Sal#47*, Sollya *Sol#5*, Butterfly pea *BpeaHF2*, pansy *BP#18*, pansy *BP#40*, Gentian *Gen#48*, Kennedia *Kenn#31*) were functional in petunia and resulted in the complementation of the *Hf1* or *Hf2* mutation in the Skr4 x SW63 petunia line (see Holton *et al.*, 1993, *supra*).

Rose

TABLE 12 Results of levels of delphinidin produced in transgenic roses using various *F3'5'H* gene expression cassettes (*CaMV 35S: F3'5'H: ocs 3'*).

F3'5'H	pCGP	Cult	#tg	TLC +	HPLC+	Highest % del	Av. % del	Northern+
Salvia2	2120	Kard	30	18/20	21/21	12%	5%	18/18
Salvia47	2119	Kard	22	11/16	9/9	7.1%	2%	12/15
Sollya	2131	Kard	27	0/23	2/2	1%	0.5%	6/6
Butterfly pea	2134	Kard	29	0/15				0/9

F3'5'H	pCGP	Cult	#tg	TLC +	HPLC+	Highest % del	Av. % del	Northern+
	pBEBF	Lav				0%	0%	
Gentian	1482	Kard	27	0/23				0/23
	pBEGH1	Lav				0%	0%	
pansy BP18	1967	Kard	56	30/33	33/34	58%	12%	21/21
	1967	SP	36	21/24	18/18	65%	35%	16/21
pansy BP40	1969	Kard	22	15/15	15/15	24%	9%	16/16
	1969	SP	37	17/17	16/17	80%	54%	11/13
	1969	Medeo	23	5/6	5/5	94%	91%	9/9
	1969	Pamela	15		4/4	90%	67%	1/1
Petunia *Hf1*	1638	Kard	22	0/21				1/17?
	1392	Lav				0%	0%	
Petunia *Hf2*	2123	Kard	41	1/27?	1/1?	nd	nd	0/10

Cult = cultivar

Kard = Kardinal

SP = Soft Promise

Lav = Lavande

#tg = # of transgenics produced

TLC+ = number of individual events that accumulated detectable delphinidin (as determined by TLC) over the number of individual events analyzed

HPLC+ = number of individual events that accumulated detectable delphinidin (as determined by HPLC) over the number of individual events analyzed

Northern = number of individual events with detectable F3'5H transcripts over the number analyzed

The cultivar Kardinal produces red colored flowers that normally accumulate cyanidin-based anthocyanins. This cultivar therefore contains functional rose F3'H and DFR activities that the introduced F3'5'H would need to compete with for substrate. The cultivar Soft Promise produces apricot colored flowers that normally accumulate pelargonidin. This cultivar is thought to lack a fully functional rose F3'H activity and contain a DFR that is

capable of acting on DHK and thus the introduced F3'5'H would only be required to compete with the endogenous rose DFR for substrate.

The results suggest surprisingly that not all of the *F3'5'H* sequences assessed (petunia *Hf1*, petunia *Hf2*, Salvia *Sal#2*, Salvia *Sal#47*, Sollya *Sol#5*, Butterfly pea *BpeaHF2*, pansy *BP#18*, pansy *BP#40*, Gentian *Gen#48*, Kennedia *Kenn#31*) were functional in rose. In fact transcripts of the introduced *F3'5'H* clones isolated from Butterfly pea, gentian, petunia *Hf1* and petunia *Hf2* failed to accumulate in rose petals. Only *F3'5'H* transcripts from pansy, salvia, kennedia and sollya accumulated in rose petals. However although Kennedia *F3'5'H* transcripts did accumulate in rose petals, there was either no accumulation of the enzyme or the enzyme produced was either not functional or was unable to compete with the endogenous rose F3'H and DFR enzymes to allow for the production of delphinidin pigments. Only the *F3'5'H* clones from salvia (*Sal#2* and *Sal#47*), pansy (*BP#18* and *BP#40*) and Sollya (*Soll#5*) resulted in the production of delphinidin based pigments in rose petals. Based on the relative percentages of delphinidin produced in rose petals, the *F3'5'H* clones from pansy (*BP#18* and *BP#40*) were revealed to be the most effective of those assessed at producing delphinidin in rose petals.

As described in the introduction, copigmentation with other flavonoids, further modification of the anthocyanidin molecule and the pH of the vacuole impact on the color produced by anthocyanins. Therefore, selection of rose cultivars with relativley high levels of flavonols and relatively high vacuolar pH would result in bluer flower colors upon production of delphinidin pigments.

The rose cultivar Medeo generally produces cream-colored to pale apricot flowers (RHSCC 158C to 159A). HPLC analysis of the anthocyanidins and flavonols accumulating in Medeo rose petals revealed that the petals accumulate high levels of flavonols (2.32 mg/g kaempferol, 0.03 mg/g quercetin) and very low levels of anthocyanins (0.004 mg/g cyanidin, 0.004 mg/g pelargonidin). The estimated vacuolar pH of Medeo petals is around 4.6.

The rose cultivar Pamela produces white to very pale pink colored flowers. It similarly accumulates low levels of anthocyanin and relatively high levels of flavonols.

The T-DNA contained in the construct pCGP1969 incorporating the pansy *F3'5'H* clone, *BP#40*, was also introduced into the rose cultivars Medeo and Pamela resulting in the production of over 90% delphinidin in these roses and leading to a dramatic color change and novel colored flowers. The most dramatic color change in transgenic Medeo flowers was to a purple/violet color of RHSCC 70b, 70c, 80c, 186b. The most dramatic color change in transgenic Pamela flowers was to a purple/violet color of RHSCC 71c, 60c, 71a, 80b.

In conclusion, two unexpected findings were revealed when gene sequences that had been proven to lead to functionality in petunia and carnation were introduced into roses.

The first was that it was not obvious which promoters would be effective in rose. Promoter cassettes that had been tested in carnation and petunia did not lead to accumulation of detectable transcripts in rose. Of the promoters tested in rose, only *CaMV 35S*, *RoseCHS 5'*, *ChrysCHS 5'*, *mas 5'* and *nos 5'* promoters led to intact and detectable *GUS* or *nptII* or *SuRB* transcript accumulation in rose.

Secondly, the petunia *F3'5'H Hf1* (and *Hf2*) sequences that had resulted in novel color production in carnation and also proven to lead to synthesis of a functional enzyme in petunia did not lead to transcript accumulation in rose petals. In fact, there was either no accumulation of detectable transcript or the transcripts that were detected were degraded and were seen as a smear or "blob" on RNA blots indicating the presence of low MW heterologous hybridizing RNA. Therefore in order to find a *F3'5'H* sequence that would accumulate in rose and lead to a functional enzyme, a number of *F3'5'H* sequences were isolated. Again it was not obvious which sequence would lead to an active enzyme in rose petals. All of the *F3'5'H* sequences isolated were tested for functionality in carnation and/or petunia and lead to accumulation of intact transcripts and production of a functional *F3'5'H* activity. However only *F3'5'H* sequences from pansy (*BP#18* and *BP#40*), salvia

(*Sal#2* and *Sal#47*) and sollya (*Soll#5*) resulted in accumulation of intact transcripts and production of a functional enzyme in rose as measured by the synthesis of delphinidin.

Table 13 shows a summary of the results obtained when assessing F3'5'H sequences from various species in petunia, carnation and rose.

TABLE 13 Summary of effectiveness of the *F3'5'H* sequences in petunia, carnation and rose

F3'5'H	Petunia		Carnation		Rose	
	Mal	RNA	Del	RNA	Del	RNA
Kennedia (*Kenn#31*)	+	*nd*	*nd*	*nd*	-	+
Gentian (*Gen#48*)	+	+	+	+	-	-
Salvia (*Sal#2*)	+	*nd*	+	+	+	+
Salvia (*Sal#47*)	+	*nd*	+	+	+	+
Sollya (*Sol#5*)	+	*nd*	+	+	+	+
Butterfly pea	+	*nd*	+	+	-	-
Pansy (*BP#18*)	+	+	+	+	+	+
Pansy (*BP#40*)	+	*nd*	+	+	+	+
Petunia (*Hf1*)	+	+	+	+	-	-
Petunia (*Hf2*)	+	+	+	+	-	-

nd = not done

Mal = malvidin detected by TLC

Del = delphinidin detected by TLC or HPLC

EXAMPLE 10

Use of pansy F3'5'H sequences in species other than rose

From the examples above, it was clear that the pansy *F3'5'H* sequences, *BP#18* and *BP#40*, resulted in functional F3'5'H activity and lead to the production of high levels of delphinidin in roses and carnations.

The T-DNA from Ti binary construct pCGP1969 (described in Example 8) containing the chimeric *CaMV 35S: pansy BP#40 F3'5'H: ocs 3'* gene expression cassette was introduced

into the gerbera cultivar Boogie *via Agrobacterium*-mediated transformation, to test the functionality of the pansy *F3'5'H* sequence in gerbera.

Of six events produced to date, one (#23407) has produced flowers with a dramatic color change (RHSCC 70c) compared to the control flower color (RHSCC 38a, 38c).

The color change of the petals of the transgenic gerbera has been correlated with the presence of delphinidin as detected by TLC.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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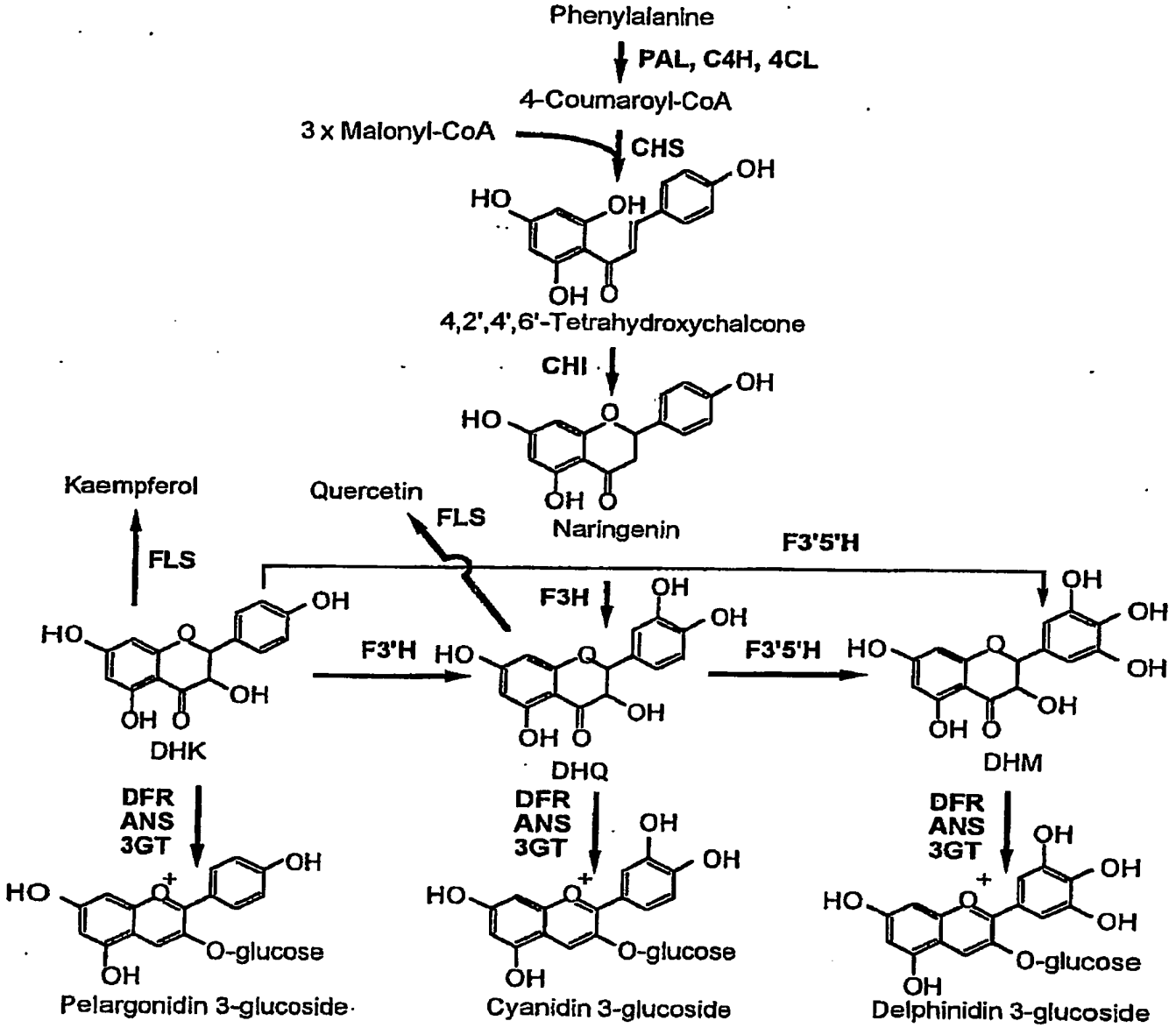
Phenylalanine
PAL, C4H, 4CL
4-Coumaroyl-CoA
3 x Malonyl-CoA
CHS
4,2',4',6'-Tetrahydroxychalcone
CHI
Naringenin
Kaempferol
Quercetin
FLS
FLS
F3'5'H
F3H
F3'H
F3'5'H
DHK
DHQ
DHM
DFR
ANS
3GT
DFR
ANS
3GT
DFR
ANS
3GT
O-glucose
O-glucose
O-glucose
Pelargonidin 3-glucoside
Cyanidin 3-glucoside
Delphinidin 3-glucoside

Figure 1A

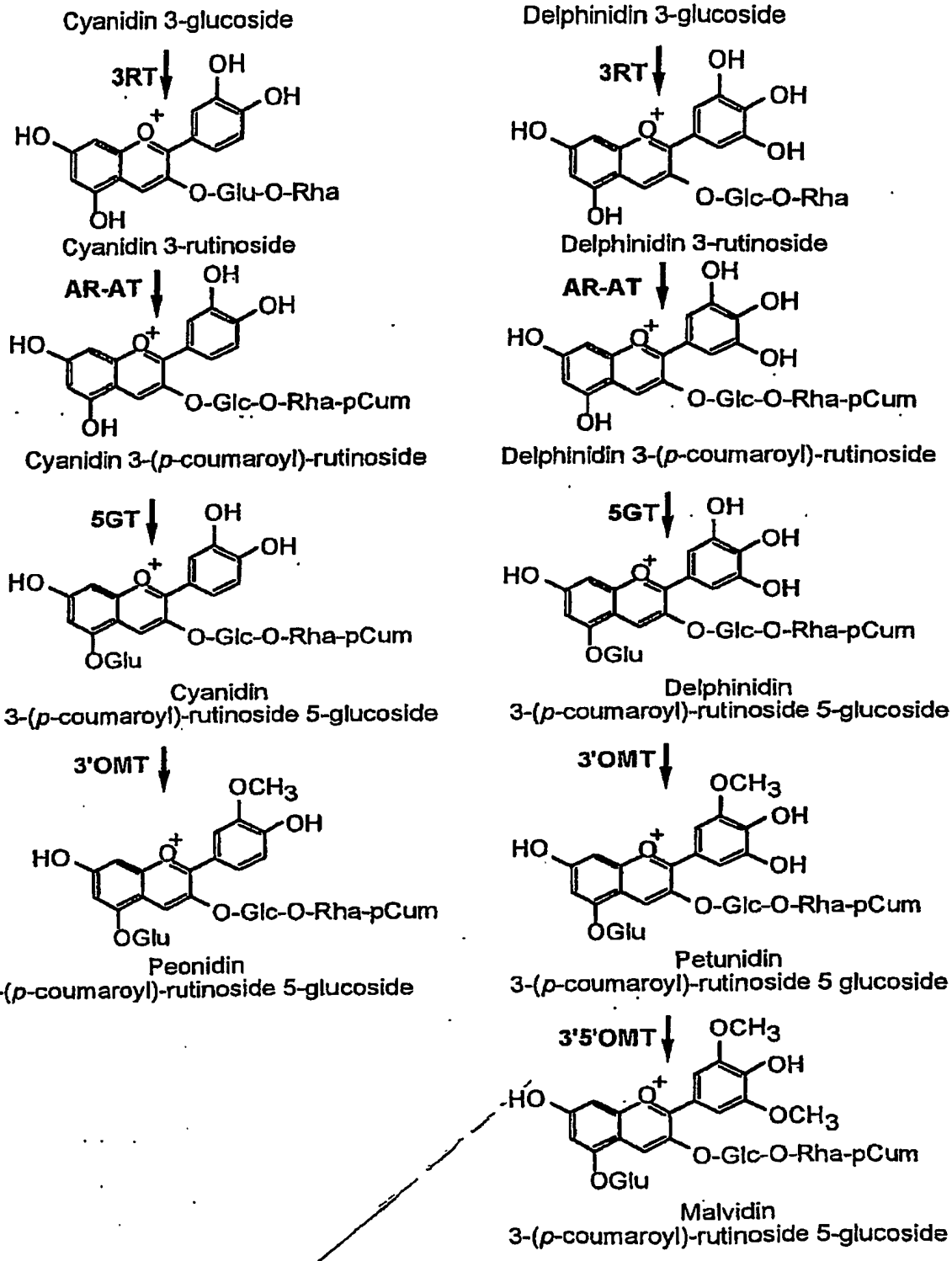
Cyanidin 3-glucoside
3RT
OH
OH
HO
O+
O-Glu-O-Rha
OH
Cyanidin 3-rutinoside
AR-AT
OH
OH
HO
O+
O-Glc-O-Rha-pCum
OH
Cyanidin 3-(p-coumaroyl)-rutinoside
5GT
OH
OH
HO
O+
O-Glc-O-Rha-pCum
OGlu
Cyanidin
3-(p-coumaroyl)-rutinoside 5-glucoside
3'OMT
OCH3
OH
HO
O+
O-Glc-O-Rha-pCum
OGlu
Peonidin
3-(p-coumaroyl)-rutinoside 5-glucoside
Delphinidin 3-glucoside
3RT
OH
OH
OH
HO
O+
O-Glc-O-Rha
OH
Delphinidin 3-rutinoside
AR-AT
OH
OH
OH
HO
O+
O-Glc-O-Rha-pCum
OH
Delphinidin 3-(p-coumaroyl)-rutinoside
5GT
OH
OH
OH
HO
O+
O-Glc-O-Rha-pCum
OGlu
Delphinidin
3-(p-coumaroyl)-rutinoside 5-glucoside
3'OMT
OCH3
OH
OH
HO
O+
O-Glc-O-Rha-pCum
OGlu
Petunidin
3-(p-coumaroyl)-rutinoside 5 glucoside
3'5'OMT
OCH3
OH
OCH3
HO
O+
O-Glc-O-Rha-pCum
OGlu
Malvidin
3-(p-coumaroyl)-rutinoside 5-glucoside

Figure 1B